The Molecular Consequences of CK2-mediated Phosphorylation of the TGA2 Transcription Factor within Systemic Acquired Resistance of *Arabidopsis thaliana*

By

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ABSTRACT

During infection, the model plant *Arabidopsis thaliana* is capable of activating long lasting defence responses both in tissue directly affected by the pathogen and in more distal tissue. Systemic acquired resistance (SAR) is a type of systemic defence response deployed against biotrophic pathogens resulting in altered plant gene expression and production of antimicrobial compounds. One such gene involved in plant defence is called *pathogenesis-related 1* (*PR1*) and is under the control of several protein regulators. TGA II-clade transcription factors (namely TGA2) repress *PR1* activity prior to infection by forming large oligomeric complexes effectively blocking gene transcription. After pathogen detection, these complexes are dispersed by a mechanism unknown until now and free TGA molecules interact with the non-expressor of pathogenesis-related gene 1 (NPR1) protein forming an activating complex enabling *PR1* transcription.

This study elucidates the TGA2 dissociation mechanism by introducing protein kinase CK2 into this process. This enzyme efficiently phosphorylates TGA2 resulting in two crucial events. Firstly, the DNA-binding ability of this transcription factor is completely abolished explaining how the large TGA2 complexes are quickly evicted from the *PR1* promoter. Secondly, a portion of TGA2 molecules dissociate from the complexes after phosphorylation which likely makes them available for the formation of the TGA2-NPR1 activating complex. We also show that phosphorylation of a multiserine motif found within TGA2's N-terminus is responsible for the change of affinity to DNA, while modification of a single threonine in the leucine zipper domain seems to be

responsible for deoligomerization. Despite the substantial changes caused by phosphorylation, TGA2 is still capable of interacting with NPR1 and these proteins together form a complex on DNA promoting *PR1* transcription. Therefore, we propose a change in the current model of how *PR1* is regulated by adding CK2 which targets TGA2 displacing it's complexes from the promoter and providing solitary TGA2 molecules for assembly of the activating complex.

Amino acid sequences of regions targeted by CK2 in Arabidopsis TGA2 are similar to those found in TGA2 homologs in rice and tobacco. Therefore, the molecular mechanism that we have identified may be conserved among various plants, including important crop species, adding to the significance of our findings.

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LIST OF ABBREVIATIONS

A - alanine

ALDI1 - AGD2-like defence response protein1

ANAC019 - Arabidopsis NAC transcription factor 19

ASF1 - Activation sequence factor 1

ATP - adenosine triphosphate

Avr gene - *avirulence* gene

AzA - azelaic acid

BTB/POZ - Broad complex, Tramtrack, and Bric a-Brac/Pox virus and Zinc finger

bZIP - basic domain/leucine zipper

CBP60g - Cam-binding protein 60-like G

CDK - cyclin-dependent kinase

ChIP - chromatin immunoprecipitation

CK2 - protein kinase CK2 (earlier casein kinase 2)

CK2α - catalytic subunit of CK2

CK2β - regulatory subunit of CK2

Cys - cysteine

D - aspartic acid

DA - dehydroabietinal

DAMP - damage-associated molecular pattern

DIBOA - 2,4-dihydroxy-1,4-benzoxazin-3-one

DIR1 - Defective in induced resistance 1 protein

DTT - dithiothreitol

E - glutamic acid

EIL1 - EIN3-like 1 protein

EIN3 - Ethylene insensitive 3

EMSA - electromobility shift assay

ERF - Ethylene response factor

ET - ethylene

FPLC - fast protein liquid chromatography

FT - Flowering locus T1

G3P - glycerol 3-phosphate

GFP - Green fluorescent protein

GTP - guanosine triphosphate

His - histidine

HR - hypersensitive response

ICS1 - isochorismate synthase 1

IPL1 - isochorismate pyruvate lyase 1

JA - jasmonic acid

JAZ - Jasmonate ZIM-domain protein

JIN1/MYC2 - Jasmonate insensitive 1/ MYC2 transcription factor

L - leucine

LS - linker scanning DNA sequences of the PR1 promoter

LS7 - DNA probe containing the LS7 sequence of the PR1 promoter

MAPK - mitogen-activated protein kinase

MeSA - methylsalicylate

MPK - MAPK kinase

MYB51 - Myb-related protein 51

NADH - reduced nicotinamide adenine dinucleotide

NADPH - reduced nicotinamide adenine dinucleotide phosphate

NahG - salicylate hydroxylase NahG

NPR1 - non-expressor of PR1-1

NPR3 - non-expressor of PR1-3

NPR4 - non-expressor of PR1-4

NRB4 - non-recognition of BTH4

OPDA - 12-oxo-phytodienoic acid

PAMP - pathogen-associated molecular pattern

PCR - polymerase chain reaction

PDF1.2 - Defensin-like protein 16

Pip - pipecolic acid

POZ - Broad complex, Tramtrack, and Bric a-Brac/Pox virus and Zinc finger

PR1 - pathogenesis-related gene 1

PR4 - pathogenesis related gene 4

qPCR - quantitative polymerase chain reaction

R gene - resistance gene

RGP - R gene product

ROS - reactive oxygen species

S - serine

SA - salicylic acid

SAR - systemic acquired resistance

SARD1 - Systemic acquired resistant deficient 1 protein

SARF - SA response factor

SDS - sodium dodecyl sulphate

SDS-PAGE - sodium dodecyl sulphate-containing polyacrylamide electrophoresis

Ser - serine

SFD1 - Suppressor of fatty acid desaturase deficiency 1 protein

SID2/EDS16 - chloroplastic ICS1 protein

SOC1 - Suppresor of constans overexpression 1 protein

T - threonine

T-DNA - Agrobacterium transfer DNA mutation

TGA - TGACG DNA-binding protein

Thr - threonine

Tyr - tyrosine

VPE - vacuolar processing enzyme

WAK1 - wall-associated receptor kinase 1

WRKY - WRKY DNA-binding protein

CHAPTER 1 - INTRODUCTION

Unlike animals, plants have several disadvantages when it comes to dealing with other, potentially harmful, species. First of all, they cannot simply change their location to avoid a certain herbivore or a pathogen outbreak. Secondly, they do not have a specialized immune system specifically dedicated to battling infectious pathogens. Regardless, plant species have endured on the face of Earth for hundreds of millions of years to the present day. So how did they do it?

Despite seeming very vulnerable to exploitation by other organisms, plants have evolved numerous preventive mechanisms enabling their survival. Some of these are structural defences. Similar to a medieval castle, a strong outer wall will foil most intrusion attempts and minimize damage to the inside environment. For this reason, the cell wall is formed by rigid layers of branched polysaccharides and lignins (Freeman and Beattie, 2008). Another fortification of the exposed above-ground tissue is the cuticle providing a second defensive layer (Muller, 2008). These structures are additionally reinforced when a potential threat is detected (Bhuiyan et al., 2009). Furthermore, various plant species synthesize a unique blend of antimicrobial compounds (Freeman and Beattie, 2008). These are chemically quite diverse, starting from very simple yet effective reactive oxygen species (such as hydrogen peroxide or the superoxide radical), continuing with slightly more complex secondary metabolites (such as antimicrobial saponins, terpenoids or alkaloids) and ending with highly complex proteins (toxic proteins and antimicrobial enzymes).

Many of these defensive countermeasures are constitutive, meaning that they are always present in the plant and prepared to deter any potential aggressor. In most cases, this is sufficient to avoid infection (Durrant and Dong, 2004). However, in the event of an attack by a strongly virulent pathogen which overpowers these defences, the plant invests its energy in enhancing inducible responses (Glazebrook, 2005). Many antimicrobial compounds are energetically costly to synthesize and, therefore, are only produced when a high-risk threat is detected. For this reason, the metabolism of cells within infected tissue is completely overhauled to limit further pathogen spread (Durrant and Dong, 2004).

As the battle rages in the infected tissue, more distant cells of the plant are informed of these events and start preparing themselves for possible disease outbreak (Glazebrook, 2005). These responses are systemic, long lasting and tailored to a particular type of threat. Biotrophic pathogens trigger systemic acquired resistance (SAR) mediated by the hormone salicylic acid, while necrotrophic pathogens feasting on dead cells induce systemic resistance facilitated by jasmonates and ethylene (Glazebrook, 2005). These hormones are perceived in distant cells where they induce defence responses via altered gene expression (Durrant and Dong, 2004). Most of these genes are directly or indirectly responsible for increasing the production of antimicrobial compounds and thus confer resistance to the pathogen.

One example of such a gene highly up-regulated during SAR is the *PR1* gene (Gruner et al., 2013). Despite the fact that its exact role remains unknown, it

is a model SAR gene the transcript of which is analysed in most studies focusing on this defence response. During biotrophic pathogen infection, there is substantial accumulation of the salicylic acid hormone in distal tissue (Durrant and Dong, 2004). This hormone is detected by its intracellular receptor, the NPR1 protein (Wu et al., 2012). A set of TGA transcription factors along with NPR1 regulate the transcription of *PR1* activating this gene during infection (Boyle et al., 2009; Rochon et al., 2006).

The focus of this thesis is to investigate the precise role of a ubiquitous protein kinase called CK2, which has been implicated in SAR (Kang and Klessig, 2005, Hildago et al., 2001), and its effect on the TGA2 transcription factor. This study provides sufficient data to incorporate CK2 into the model of *PR1* regulation via TGA2 and NPR1.

1.1 Outline

This thesis begins with a literature review (Chapter 2) of the current knowledge of plant defence mechanisms in general and later focuses on responses triggered by biotrophic pathogens. These include the localized hypersensitive response and systemic acquired resistance (SAR). Of particular interest are the downstream SAR molecular components such as the *PR1* gene and its regulators; the TGA2 and NPR1 proteins. Finally, the currently known facts about protein kinase CK2, its function and its role in SAR are also presented.

Chapter 3 is a description of the methods used to analyse how CK2 affects TGA2 and the physiological role of this event in the Arabidopsis plant. Chapter 4 provides a detailed account of the obtained results identifying which regions of TGA2 are targeted by the kinase and demonstrating the importance of this modification on this transcription factor's interactions with other proteins or DNA molecules. The relevance of these results is discussed in Chapter 5, which looks into how our data resolves several issues encountered by previous research studies, how phosphorylation affects several TGA2 domains leading to diverse effects on intermolecular interactions and how our findings may be transferable to other plant species.

Finally, Chapter 6 concludes this thesis and identifies several avenues worth exploring in future research enabled by the findings of this study.

CHAPTER 2 - LITERATURE REVIEW - PLANT DISEASE RESPONSES AND THE PROTEIN KINASE CK2

2.1 Plant defence mechanisms

Plants are a valuable source of energy and nutrients for a wide array of heterotrophic organisms. In order to survive in potentially hostile environments, plants have evolved numerous countermeasures against both herbivores and pathogens. Some of these defence mechanisms are fairly crude yet provide constant protection against a variety of harmful species, while others are incredibly complex systems specific to a particular type of threat, tightly regulated and coordinated with other cellular mechanisms. Although they are not comparable with the human adaptive immune system, their efficiency has helped plants endure millions of years of cohabitation with evolving pathogen and herbivore species.

2.1.1 Constitutive defences

Most microorganisms are deterred from harming a plant by a series of permanent defences, including structural, chemical and proteinaceous deterrents, which can be surpassed only by more specialized pathogens.

2.1.1.1 Structural defences

There are multiple physical barriers shielding plant cells from harmful organisms. The cell wall is the first line of defence against both herbivores and pathogens although its main role is support of cellular structures and maintenance of turgor pressure. The cell wall is composed mostly of cellulose

fibres cross-linked by branched polysaccharides for additional mechanical strength. Furthermore, neighbouring cells have walls "cemented" together by hydrated gels made of pectin, which seal any openings and thus prevents pathogen intrusion.

In order to further fortify the outer plant cells, many cell walls go through a process called lignification. Lignin is a complex phenolic biopolymer and the primary compound of wood. It connects branched hemicellulose molecules giving the plant cell additional rigidity. In general, lignified cells are highly impermeable to pathogens and tougher for smaller herbivorous insects to chew (Freeman and Beattie, 2008). Lignification also renders the cell walls more resistant to water and, therefore, less susceptible to fungal degradation enzymes (Nicholson and Hammerschmidt, 1992). Additionally, further lignification of cell walls is a direct response to infection by biotrophic fungi; plants assemble cell wall appositions known as papillae made of lignin and similar phenolic compounds at the sites of attempted penetration by powdery mildew fungus (Bhuiyan et al., 2009).

All above-ground plant tissue is further protected by another physical barrier; a translucent film of polymeric lipids and waxes known as the cuticle. Its precise composition varies among plant species, however, the major cuticle components are the biopolyester cutin, very long-chain fatty acids and their derivatives, and a mix of cyclic compounds such as pentacyclic triterpenoids (Muller, 2008). This hydrophobic layer is efficient in inhibiting attachment, locomotion and feeding of many heterotrophic organisms.

2.1.1.2 Constitutive chemical deterrents

Production of antimicrobial secondary metabolites greatly affects the plant's susceptibility or resistance to specific pathogens. Many such molecules are synthesized in advance during normal plant development and are stored in the most exposed tissues in specialized organs. These preformed chemicals are called phytoanticipins as they are produced in anticipation of an infection (Van Etten et al., 1994). For example, saponins are glycosylated triterpenoids or steroids exhibiting soap-like properties. These compounds can be incorporated into plant cell membranes and disrupt those of invading fungal or bacterial pathogens (Soetan et al., 2006). Oat roots produce a saponin called avenacin which hinders penetration and growth of the fungus *Gaeumannomyces graminis*. This is due to avenacin's ability to form complexes with sterols found in the fungal membrane leading to loss of membrane integrity (Keukens et al., 1995). Infection of the root can occur only if the pathogen can produce a hydrolase detoxifying avenacin by removing the sugar moiety of the molecule (Crombie et al., 1986) or if the pathogen lacks membrane sterols altogether (Morrissey and Osbourn, 1999).

Another phytoanticipin example is found in tomato. α-tomatine is a constitutively produced steroidal glycoalkaloid implicated in plant defence. Its concentration in tomato leaves is sufficient to inhibit fungal and non-pathogenic microbe growth (Arneson and Durbin, 1968). However, some pathogens such as *Botrytis cinerea* have evolved detoxifying enzymes capable of hydrolyzing sugars from α-tomatine (Bednarek and Osboun, 2009).

A different approach can be observed during infection of rye when the hydroxamic acid 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) is hydrolysed by an activated enzyme and further decomposes into smaller fragments which exhibit fungistatic and bacteriostatic activity (Niemeyer, 1988). Another tactic is utilized by *Arabidopsis thaliana* which produces glucosinolate phytoanticipins (Stotz et al., 2011). These sulphur-rich compounds are spatially separated from myrosinase enzymes but are brought into contact by tissue damage. The resulting hydrolysis products, such as thiocyanates and epithionitriles, are highly toxic for invading pathogens (Lambrix et al., 2001).

2.1.1.3 Proteinaceous pesticides

Apart from utilizing small molecules against microbes and herbivores, plants have another effective weapon in their arsenal: proteins. Based on their effect, proteins as direct antimicrobial or anti-herbivore agents can be divided into toxins, digestive enzyme inhibitors or hydrolytic enzymes.

Studies of the castor bean (*Ricinus communis*) and jequirity bean (*Abrus precatorius*) discovered highly toxic proteins ricin and abrin (Olsnes et al., 1974). Their lethal effect is attributed to inactivation of the 60S ribosomal subunit by cleaving a particular N-glycosidic bond of 28S rRNA thus effectively disrupting proteosynthesis in all eukaryotic cells (Endo and Tsuguri, 1987).

The second group of proteins utilized for pest control are inhibitors of digestive enzymes. Plant serine protease inhibitors often function as pseudo-substrates, tightly binding to the active site of serine proteases such as trypsin

and chymotrypsin (Bode and Huber, 1992). This stops the pathogen's enzyme from performing its biological role and limits the organism's metabolism. For example, a trypsin inhibitor isolated from corn is detrimental to conidium germination and growth of multiple pathogenic fungi including *Aspergillus flavus* and *Fusarium moniliforme* (Chen et al, 1999).

The last major group of antimicrobial proteins are hydrolases targeting polymeric compounds, such as chitin, peptidoglycan and β-1,3-glucans, associated with certain types of microbes. Chitinases catalyze the cleavage of the β-1,4-glycoside bond of chitin, a natural polymer composed of β-1,4-linked N-acetyl glucosamine residues found abundantly in cell walls of fungi and bacteria (Li and Rossman, 2004). Additionally, some chitinases have lysozyme-like activity meaning that they are capable of hydrolysing another bacterial structural polymer, peptidoglycan (Kasprzewska, 2003). Localized outside the plasma membrane and thus close to infection sites, apoplastic chitinases cause lysis of hyphal tips, effectively blocking further fungal growth (Grover, 2012). Furthermore, the breakdown products of fungal chitin serve as signaling molecules for triggering long-lasting systemic plant defence responses (Kasprzewska, 2003). These induced countermeasures also include production of a second, more effective, group of chinases stored in vacuoles and released only during cell penetration by fungal hyphae (Kasprzewska, 2003). In general, the ability to produce chitinases in sufficient amounts correlates with plant resistance to various pathogens (Shrestha et al., 2007).

2.1.2 Localized responses to pathogen infection

In some cases, the aforementioned constitutive defences may not be sufficient to deter a particular pathogen. Therefore, the plant responds to such a threat by up-regulating and strengthening some constitutive countermeasures and also by inducing a new array of responses, such as producing additional antimicrobial compounds or initiating the hypersensitive response.

2.1.2.1 Inducible phytoalexins

Another group of small antimicrobial compounds produced by plants are phytoalexins. Unlike the constitutively synthesized phytoanticipins, phytoalexin production occurs only after contact with a pathogen (Van Etten et al., 1994). They are biosynthesized *de novo* and transported to the site of infection. Being usually less potent than phytoanticipins, phytoalexins work in a concentration-dependent manner. Produced in both disease-resistant and susceptible plants, these compounds accumulate to higher levels and more rapidly in resistant varieties especially at the sites of infection (Morrisey and Osbourn, 1999). Phytoalexin degradation capabilities directly correlate to pathogenicity of various fungi (Van Etten et al., 1989).

Phenolic stilbenes are synthesized by grapevines in response to fungal challenge. These compounds, such as resveratrol, display antifungal activity against an array of fungal pathogens (Morrisey and Osbourn, 1999). Stilbene accumulation levels positively correlate with resistance of vine varieties (Sarig et al., 1997) and the ability of *Botritys cinerea* to infect these plants is attributed to its stilbene degradation capability (Sbaghi et al., 1996).

The resistance of *Arabidopsis thaliana* to *Alternaria* species has been credited to the production of camalexin, the major phytoalexin found in this plant (Tsuji et al., 1992). Camalexin is structurally similar to the widely used synthetic fungicide thiabendazole which binds to the respiratory complex II inhibiting ubiquinone reduction thus blocking cellular respiration (Zhou et al., 2011).

Due to the fact that the categorization of molecules into phytoalexins or phytoanticipins is based on their induced or constitutive production, there are antimicrobial compounds that fall into both categories. For example, maackain is a preformed isoflavonoid derivative found in red clover. It is stored in its glucosylated form and released during tissue damage. However, it is also newly synthesized after infection or elicitor treatment (Van Etten et al., 1994).

2.1.2.2 Reactive oxygen species

Interaction of pathogens with the host plant can also trigger accumulation of reactive oxygen species (ROS). The primary ROS include superoxide, hydrogen peroxide, the hydroxyl radical and nitric oxide (Daudi et al., 2012). These molecules are highly reactive causing oxidative destruction of close cellular components (Asada and Takahashi, 1987). Their rapid production near infection sites is directly toxic to pathogens (Liu et al., 2010). This effect is known as the oxidative burst and occurs in two phases: the first is rapid but weak ROS generation by enzymes present in the cell wall or plasma membrane at the time of contact with pathogen, while the second phase is characterized by massive and continuous ROS production by *de novo* synthesized enzymes (Yoshioka et al., 2008).

In the first phase, most ROS molecules are produced either by apoplastic peroxidases or membrane-associated NADPH/NADH-dependent oxidases (Liu et al., 2010). Microbial virulence factors, such as the bacterial flagellin protein or elicitors derived from hydrolysed β-1,3-glucans and chitin (Daudi et al., 2012), serve as signals and their perception is immediately followed by a cytosolic influx of Ca^{2+} and an apoplastic rise of pH (Grant et al., 2000; Fellbrich et al., 2000). Increased Ca^{2+} levels are sensed by calcium-dependent protein kinases which activate membrane-associated oxidases via phosphorylation (Yoshioka et a., 2008), while extracellular alkalinization activates peroxidases in the plant cell wall (Bolwell et al., 2002). The crucial importance of such enzymes was demonstrated in Arabidopsis transgenic plants. The lack of two identified peroxidases compromised the plant's ability to perform an oxidative burst making it susceptible to a number of pathogens (Daudi et al., 2012). Furthermore, the activation of signaling cascades after pathogen infection also leads to increased biosynthesis of ROS-producing enzymes (Yoshioka et al., 2008).

Due to the fact that ROS molecules do not discriminate between pathogen and host targets, the localization of their production outside of the plant cells is highly important. To minimize the negative impact on non-infected host tissue, plant cells also up-regulate biosynthesis of cytosolic scavenging enzymes, such as the superoxide dismutase or catalase, metabolizing ROS into less harmful compounds (Liu et al., 2010).

2.1.2.3 The hypersensitive response

Necrosis of plant tissue as a result of biotrophic pathogen exposure has been studied for almost an entire century. Coined by E.C. Stakman in 1915, the hypersensitive response (HR) was described as "an abnormally rapid host plant death when attacked by rust fungi" (Stakman, 1915). Since then, HR has been observed in most of the studied plant species as a result of infection by a myriad of diverse biotrophic pathogens including viruses, bacteria, fungi and oomycetes (Mur et al., 2008). The entire process is quite rapid. For example, cell death in potato cultivars has been observed within 24 hours after inoculation with the fungus *Phytophthora infestans* (Vleeshouwers et al., 2000).

The first studies of HR were cytological, focusing on what happens to the plant cell after pathogen detection. Much insight into the process was obtained using live video microscopy. This method enabled the analysis of a single cell HR occurring during potato or parsley cell interaction with *P. infestans* (Gross et al., 1993; Freytag et al., 1994). After attempted penetration by the fungal intracellular infection hyphae, there was an immediate apposition of phenolic compounds and callose reinforcing the cell wall (Freytag et al., 1994). However, when the pathogen was successful in forming an intracellular vesicle necessary for infection, the plant cell underwent HR in order to suppress further damage. The host cytoplasm took on a granular appearance and aggregated around the pathogen’s vesicle. This was followed by the rapid expansion of the cytoplasm and nucleus resulting in a sudden collapse of both and ended in the collapse of the pathogen's intracellular vesicle (Freytag et al., 1994).

Other research groups focused on HR at the molecular level. The detection of a threat is mediated by gene-for-gene interactions. These interactions simplistically explain how the inheritance of the host plant's resistance and the pathogen's virulence is controlled by a pair of genes (Keen, 1990). The presence of a microbial *avirulence* (*avr*) gene results in the recognition by certain plant species or cultivars via their *resistance* (*R*) gene. *Avr* gene products lead directly or indirectly to the production of elicitors which, detected by *R* gene products (RGPs), trigger plant defences such as the HR (Keen, 1990). Should a plant not have such a gene, it would be susceptible to microbial infection.

Several pathways in which the detected signal is transmitted from the receptor proteins have been described. For example, an entire class of RGPs physically interact with proteins forming the mitogen-activated protein kinase 4 signaling complex involved in triggering generation of reactive oxygen species (Mur et al., 2008). Another study has shown that some RGPs indirectly interact with components of ubiquitin ligase (Azevedo et al., 2002). It has been hypothesized that this process could lead to ubiquitination and subsequent degradation of cell death suppressors thus initiating the HR (Mur et al., 2008).

Among such suppressors could be calcium channel blockers. As in mammalian cell death, rapid cytoplasmic calcium influxes have been measured after application of *avrD* gene product, *Pseudomonas syringae* bacteria and rust fungus on various plant species (Atkinson et al., 1996; Grant et al., 2000; Xu and Heath, 1998). Additionally, treatment with the calcium channel blocker $LaCl_3$

suppresses HR (Xu and Heath, 1998). Therefore, elevated calcium levels are a crucial prerequisite for ROS production leading to an oxidative burst and HR (Grant et al., 2000).

The most detrimental effect of high ROS concentrations during cell death can be seen on cellular and organelle membranes. Hydroxyl radicals extract hydrogen atoms from lipids triggering chain peroxidation reactions. Peroxidation of unsaturated membrane lipids greatly affects their shape and thus disrupts the membrane's integrity (Mur et al., 2008). *In vivo* analyses have shown this reaction occurring during HR (Kenton et al., 1999). Increasing cytoplasmic Ca^{2+} and ROS concentrations also affect mitochondria and chloroplasts. Elevated mitochondrial ROS levels destabilize its membrane resulting in the formation of permeability transition pores and mitochondrial swelling (Moller, 2001). The inevitable result is the dissipation of mitochondrial membrane potential and disruption of electron flow (Moller, 2001). Without properly functioning mitochondrial respiration, cellular ATP levels plummet disrupting anabolism (Xie and Chen, 2000). In the end, cytochrome C is released from respiratory enzymes, a hallmark of plant HR (Krause and Durner, 2004).

On the other hand, ROS production leading to lipid peroxidation in chloroplasts seems to be light-dependent (Montillet et al., 2005). A rapid decrease of internal CO_2, possibly due to stomatal closure, results in a surplus of absorbed energy which cannot be consumed by the dark reactions of photosynthesis. Chlorophyll with P680 reaction centres becomes over-excited and transfers the energy to oxygen creating highly reactive singlet oxygen which

reacts with chloroplast membranes changing their appearance within several hours after HR induction (Mur et al., 2008). The photosynthetic electron flow is reduced and together with a decrease in chlorophyll fluorescence is indicative of photosystem damage (Almeras et al., 2003). Furthermore, chlorophyll is released from photosynthetic proteins and catabolised into often toxic porphyrin compounds (Szabo et al., 2005).

Fragmented material and remnants of organelles are transported into large vesicles emerging in the cytoplasm at later HR stages (Mittler et al., 1997). These vesicles are of vacuolar origin and their purpose is probably the same as in autophagy (the degradation of dysfunctional cellular components). These vesicles contain vacuolar processing enzymes (VPEs), proteases contributing to vacuolar processing and also acting as crucial effectors in HR. Decreasing the amount of VPEs via gene silencing significantly reduced or even completely compromised HR cell death (Hatsugai et al., 2004).

Vesicular formation is also important in localizing the HR only to infected and neighbouring cells thus preventing runaway cell death. Production of proteins involved in the formation of vacuolar vesicles is induced during HR, while their decreased proteosynthesis via gene silencing results in runaway cell death around pathogen-induced lesions (Liu et al., 2006). Therefore, it has been proposed that autophagy of disrupted cellular components by these vesicles is crucial for containing HR effectors inside the dying cell for as long as possible decreasing their negative effects on non-infected cells (Liu et al., 2006).

In the end, the internal destruction of mitochondria, chloroplasts and vacuoles leads to cytoplasmic and nuclear collapse and the subsequent collapse of invading pathogen cells due to the accumulation of cytotoxic compounds (Freytag et al., 1999).

2.1.3 Systemic responses to pathogen infection

Despite all the aforementioned countermeasures deployed in the vicinity of infection, the threat of further disease spread still looms over the host plant. For this reason, plant cells in distal tissue are informed about the infection via mobile signals and altered hormone levels resulting in preventative up-regulation of defence mechanisms. Due to the diversity of plant pathogens, multiple defence pathways exist at the molecular level. Modulation of activity and cross-talk between these pathways are essential for providing the best outcome halting disease spread.

Recognition of a necrotrophic pathogen infection triggers the jasmonic acid/ethylene (JA/ET) pathway in which these two molecules accumulate in plant tissue. Acting as hormones, their increased levels result in activation of a particular set of defence responses targeting the necrotrophic pathogen. Components of this pathway are also utilized during wounding and insect herbivory demonstrating the cooperation and co-regulation of certain defence pathways.

On the other hand, infection by pathogens needing live host cells for their growth and development results in the production of salicylic acid (SA) in distal

plant tissue. As a result, measures specific to fight such biotrophic organisms are deployed leading to systemic acquired resistance (SAR). However, the differentiation of pathogens into the 2 groups based on their nutrient source requirements is not so clear-cut, since some species switch from one to the other at various stages of their life cycle. These so-called hemi-biotrophs also elicit systemic plant responses that are tailored to this specific threat (Glazebrook, 2005).

The capability of mounting a systemic resistance is crucial for plant survival in hostile environments. However, the process is energetically very expensive and, therefore, cannot be maintained constitutively throughout the entire life cycle of a plant (Heil et al., 2002).

2.1.3.1 Induced systemic resistance to necrotrophic pathogens

Necrotroph infection causes the up-regulation of antimicrobial defences throughout the entire plant organism. Detection of this sort of pathogen is not mediated by gene-for-gene interactions and, therefore, the hypersensitive response (HR) is not triggered (Glazebrook, 2005). Due to the fact that necrotrophs uptake their nutrients from dead cells, HR leading to host cell death would be counterproductive. In fact, HR activation using elicitors enhances colonization by necrotrophic fungus (Govrin and Levine, 2000). This type of pathogen typically deploys an array of cell wall-degrading enzymes and phytotoxic compounds leading to host cell necrosis facilitating nutrient uptake (Mengiste, 2012). All host-specific necrotrophs produce host-specific toxins which are essential for virulence and thus limit infection to susceptible host genotypes

(Wolpert et al., 2002). Unlike gene-for-gene interactions, resistance to such toxins is achieved using multiple approaches. For example, the production of detoxifying enzymes, presence of toxin-insensitive alleles displaying low recognition specificity or enhanced cellular defence mechanisms limiting necrosis (Wolpert et al., 2002). Therefore, numerous genes are required to decrease susceptibility to a host-specific necrotroph.

Fungi such as *Botritys cinerea* and *Alternaria brassicicola* or the *Erwinia carotovora* bacteria are broad host-range necrotrophs. Their virulence is not mediated by the production of a host-specific toxin. However, their recognition is achieved via pathogen-associated or damage-associated molecular patterns (PAMPs and DAMPs, respectively). Bacterial flagellin, secreted pathogen enzymes or fungal cell wall fragments are a few examples of PAMPs, while pathogen-induced plant cell wall degradation products, such as oligogalacturonides or cutin monomers released from the cuticle, are typical DAMPs (Boller and Felix, 2009). These compounds are detected by membrane-localized receptors often acting as or interacting with transmembrane kinases. One example is the wall-associated kinase 1 protein (WAK1) acting as an oligogalacturonide receptor (Brutus et al., 2010). Necrotroph-produced polygalacturonases target plant pectin releasing oligogalacturonides. These interact with the extracellular domain of WAK1 and activate its intracellular kinase domain transmitting the signal via protein phosphorylation (Brutus et al, 2010). Of note, phosphorylation resulting in activation of several mitogen-associated protein kinases (MPK3 and MPK6) is a point of convergence downstream of

PAMPs and DAMPs (Tsuda and Katagiri, 2010). Additionally, this transduction pathway responds only to necrotrophic pathogens and displays minimal activity during biotrophic interactions (Mengiste, 2012).

Nevertheless, several similar cellular responses as in the HR are detected after signal recognition. Ion fluxes, medium alkalinization, cell wall lignification and oxidative bursts utilizing ROS are common denominators for both biotroph-triggered HR and early anti-necrotroph responses (Ferrari et al., 2007). ROS molecules are an important part of the HR, acting as antimicrobial and signaling compounds regulating cell death and greatly affecting biotrophic pathogens. Amazingly, ROS seem to be not only tolerated but are actually generated by certain necrotrophs during infection and thus act as virulence factors (Temme and Tudzynski, 2009). Pathogenicity and the aggressiveness of *Botrytis cinerea* strains are directly dependent on the concentration of hydrogen peroxide and superoxide radicals present in leaf tissue during infection (Tiedemann, 1997).

Despite the similarities, necrotrophic infection results in the alteration of specific phytohormone levels, most importantly the increase of jasmonic acid (JA) and ethylene (ET). Interestingly, components of the JA/ET pathway are also activated during osmotic stress, drought, wounding or exogenous application of elicitors, such as chitins or oligosaccharides (Turner, 2002). JA and related molecules called jasmonates are lipid-based phytohormones that also play important roles in physiological processes like root growth, pollen development, senescence or fruit ripening (Lorenzo and Solano, 2005). The initial rapid and transient increase of JA and its precursor 12-oxo-phytodienoic acid (OPDA) after

leaf infection is attributed to the enzymatic activity of a lipase (Stelmach et al., 2001). This enzyme releases OPDA from chloroplast membranes where it is endogenously stored. The initial JA increase activates five genes encoding for JA biosynthesis enzymes (Turner et al., 2002). These enzymes are localized to the chloroplast where they convert the abundant linolenic acid into OPDA. This crucial JA precursor is subsequently transported into the peroxisome where it undergoes reduction and β-oxidation reactions to form JA or other jasmonates (Turner et al., 2002). Local JA accumulation leads to rapid JA increases in the phloem of infected leaves (Truman et al., 2007). Enhanced levels have also been observed in systemic leaves suggesting that JA or one of the jasmonates are the mobile signal in this systemic plant immune response (Chaturvedi et al., 2008).

The JA signal affects gene expression via degradation of repressors or activation by transcription factors. Jasmonate ZIM-domain (JAZ) proteins interact with the JIN1/MYC2 transcription factor and thus inhibit transcription. JA promotes interaction of JAZ proteins with ubiquitin ligases marking them for subsequent proteosomic degradation (Chini et al., 2007). JIN1/MYC2 is thus released and can activate JA-responsive genes (Lorenzo et al., 2004). Genetic studies have identified WRKY33 and MYB51 as additional transcription factors regulating immune responses to necrotrophic fungi (Mengiste, 2012).

Overall, 41 JA-responsive genes have been found in Arabidopsis using microarray analysis (Sasaki et al., 2001). While five are important for JA biosynthesis, others are responsible for production of antimicrobial compounds such as camalexin and indol glucosinolates, production of antimicrobial enzymes

like β-1,3-glucanase or chitinase, or regulate cell wall biosynthesis (Mengiste, 2012). Therefore, changes in gene expression induced by JA in local and distal tissue significantly contribute to systemic resistance against necrotrophic pathogens.

Ethylene (ET) is also a plant hormone involved in many physiological processes including flower senescence, ripening and wound responses (Van Loon et al., 2006). Its importance in plant defence responses was clearly demonstrated when it was exogenously applied to plants (Beckmann, 2000). During infection, virulence factors or elicitors trigger *de novo* ET production from methionine sequentially metabolised into S-adenosinemethionine, 1-aminocyclopropane-1-carboxylic, acid and finally ET (Adie et al., 2007). Biosynthetic enzymes are tightly regulated at the transcriptional and post-translational levels. Similarly as in the JA pathway, protein phosphorylation by calcium-dependent and mitogen-activated protein kinases is crucial in this regulatory process (Liu and Zhang, 2004). Once produced, ET is responsible for activation of transcription factors known as ethylene response factors (ERFs; Ohme-Takagi and Shinshi, 1995). These bind to a regulatory element called the GCC box found in promoters of defence-related genes like *PDF1.2* and *PR4* (Van Loon et al., 2006).

It is generally accepted that JA and ET act together in response to necrotrophs and antagonize salicylic acid induced responses (Van Loon et al., 2006). However, this is an oversimplified view. For example, Arabidosis resistance to necrotrophic *A. brassicicola* is mediated by the JA pathway alone

without the help of ET (Glazebrook, 2005). Therefore, it seems that these pathways are fine-tuned to provide a characteristic response to a specific necrotrophic pathogen.

2.1.3.2 Systemic acquired resistance against biotrophs

The second major type of systemic disease resistance in plants is systemic acquired resistance (SAR). This long-lasting defence response is effective against biotrophic and hemibiotrophic pathogens and requires a functional salicylic acid (SA) signaling pathway. As described previously, detection of biotrophic pathogens is typically mediated via gene-for-gene interactions (Dangl and Jones, 2001). This interplay of pathogen *avirulence* (*avr*) genes and host *resistance* (*R*) genes leads in most cases to the hypersensitive response (HR), the rapid necrosis of plant cells in the vicinity of infection sites (Mur et al., 2008). These cells are sacrificed to contain the biotrophic pathogen. However, the possibility of further disease spread along with potentially increased pathogen presence in the environment necessitates systemic precautions. For these reasons, mobile signals trigger SAR in distant tissue via elevated SA levels resulting in activation of *pathogenesis-related* (*PR*) genes (Durrant and Dong, 2004). Such strong countermeasures are highly effective against a broad range of biotrophic pathogens and can protect a plant for weeks to months (Fu and Dong, 2013).

2.1.3.2.1 Mobile SAR signals

Elevated concentrations of SA in both proximal and distal tissues after infection are a hallmark of SAR (Fu and Dong, 2013). Early studies tested if SA

itself could be the mobile signal travelling through plant vasculature and informing uninfected cells of a threat. However, this hypothesis was rejected using grafting experiments with wild type and *nahG* mutant tobacco plants. These mutants express the bacterial *nahG* gene encoding a salicylate hydroxylase capable of turning SA into catechol resulting in the inability of *nahG* plants to accumulate free SA and trigger SAR (Ryals et al., 1996). Grafting studies demonstrated that SAR activation was successful in chimeric plants containing a wild type scion grafted onto a *nahG* rootstock (Vernooij et al., 1994). Conversely, plants containing a wild type rootstock and an SA-deficient scion failed to mount a SAR. Therefore, systemic defence is achieved by systemic SA production and not SA transport from infection sites.

The mobile signal is most likely conducted by the phloem since SAR signaling was disrupted after removal of phloem tissue in the stem above the infection site (Tuzun and Kuc, 1985). Additionally, treatment using phloem sap-enriched petiole exudates obtained from inoculated plants is sufficient to induce systemic resistance in non-infected plants (Maldonado et al., 2002)

Methyl salicylate (MeSA), created by enzymatic methylation of SA, has also been investigated as a putative long-distance signal. Elevated MeSA concentrations in leaf tissue and petiole exudates of inoculated plants, suppression of SAR in mutants lacking MeSA biosynthetic genes, as well as chemical inhibition of MeSA esterases compromising SAR, all support a potential signaling function (Dempsey and Klessig, 2012). Amazingly, the extent to which MeSA influences SAR seems to be dependent on the length of light exposure

after pathogen inoculation (Liu et al., 2011). Arabidopsis mutants incapable of MeSA accumulation did not mount SAR if they were inoculated late in the day. However, a full response was detected after inoculation in the morning. Furthermore, other studies have outright dismissed the essential role of MeSA in SAR signaling demonstrating that the majority of this volatile compound is emitted into the atmosphere where it possibly serves as a signal for other plants informing them of pathogen presence (Attaran et al., 2009).

Not just small molecules but also proteins could act as the mobile signal. Genetic screening of SAR-deficient T-DNA insertion lines identified the *defective in induced resistance 1-1* (*dir1-1*) gene mutant (Maldonado et al., 2002). Although this mutant recognizes both virulent and avirulent pathogens leading to local defence responses, it is unable to systemically induce *PR* genes. Subsequent studies showed that the gene`s product, the DIR1 protein, is a lipid transfer protein capable of binding two molecules of long-chain fatty acids (Lascombe et al., 2008). Additionally, it contains protein-protein interaction domains which could interact with a possible receptor in distal tissue. However, DIR1 may be just a carrier for the signal. Petiole exudates from *dir1-1* mutants cannot trigger SAR but the mutant will respond to exudates from inoculated wild type plants suggesting a potential DIR1 role in either synthesis or transport of lipid molecules acting as the signal (Maldonado et al., 2002).

A similar story can be seen in studies dealing with the *suppressor of fatty acid desaturase deficiency 1* (*sfd1*) mutant. It also fails to develop SAR after pathogen inoculation but petiole exudates from inoculated wild type plants can

restore SAR in this mutant (Chaturvedy et al., 2008). The *sfd1* gene encodes a reductase catalyzing NADH-dependent reduction of dihydroxyacetone phosphate to glycerol-3-phosphate (G3P). This compound is required for proper lipid metabolism and for SAR and exogenous application of G3P on *sfd1* plants restores SAR after infection (Chanda et al., 2011). Nevertheless, G3P treatment by itself will not induce SA production or SAR. Additionally, G3P mobility seems to be dependent on the presence of DIR1 and vice versa, since this protein fused to the green fluorescent protein (GFP) is not systemically transported in the *sfd1* mutant (Chanda et al., 2011).

Another small molecule requiring DIR1 for its role in SAR signaling is azelaic acid (AzA). Concentrations of this C9 dicarboxylic acid are up-regulated in petiole extracts during SAR (Jung et al., 2009). Localized exogenous treatment of Arabidopsis plants using radioactively labelled AzA resulted in elevated radioactivity levels in distal tissue when exposed to *P. syringae*. But once again, exogenous application alone did not trigger SA accumulation or expression of *PR* genes. It seems that AzA is more involved in priming plant immunity resulting not in direct defence activation but rather in quicker responses to a subsequent infection (Jung et al., 2009).

Furthermore, the diterpenoid dehydroabietinal (DA) is a potent SAR-activating compound (Chaturvedy et al., 2012). It is also dependent on DIR1 during SAR and acts synergistically with AzA. Radioactively labelled DA was quickly transported from sprayed to distal tissue leading to increased levels of SA and *PR* gene transcripts.

Based on all this information, it would seem that there might not be a solitary signaling molecule triggering defences in distal tissue but that this response could be mediated by the interplay of various factors like DIR1, G3P, AzA, DA and possibly others. This hypothesis could explain how a plant is able to modulate systemic resistance against various types of biotrophic pathogens.

This question could also be answered by the existence of multiple signaling pathways. Recent studies have identified high levels of pipecolic acid (Pip) in petiole exudates, and in local and systemic leaves after pathogen inoculation (Navarova et al., 2012). Pip is a non-protein amino acid and a common catabolite of lysine in plants. Its biosynthesis involves a transamination step catabolised by AGD2-like defence response protein1 (ALDI1) and this enzyme is necessary for Pip accumulation post infection. Exogenous Pip treatment of *aldi1* mutants restores pathogen detection and SAR activation. Such treatment of wild type plants results in increased resistance. Thus far, no direct connection between Pip and the other mobile signal candidates has been found (Dempsey and Klessig, 2012).

2.1.3.2.2 Systemic SA production after signal perception

Mobile signals released by infected cells must be perceived in distant tissue in order to successfully activate SAR. Despite the identification of potential molecular signals, their receptors have not been discovered. Signal perception results in systemic salicylic acid (SA) accumulation and subsequent expression of *pathogenesis-related* (*PR*) genes.

It was initially thought that SA in plants is synthesized by the shikimate-phenyl propanoid pathway, which turned out to be incorrect (Durrant and Dong, 2004). In bacteria, SA is produced by 2 enzymes: the isochorismate synthase 1 (ICS1) and the isochorismate pyruvate lyase 1 (IPL1). ICS1 converts chorismate to isochorismate which is subsequently turned over into SA by IPL1 (Durrant and Dong, 2004). Arabidopsis mutants *sid2* and *eds16* are incapable of SA accumulation after pathogen challenge. Characterization of the SID2/EDS16 protein successfully identified a chloroplastic ICS1 similar to the bacterial homolog, and involved in SA production during plant SAR (Wildermuth et al., 2001). SA levels during pathogen infection in *sid2* and *eds16* plants are substantially reduced to about 5-10 % of wild type levels. The remaining SA is most likely synthesized from cinnamate; however, this metabolic pathway seems to have a minor role in SAR (Chen et al., 2009). Unfortunately, the plant IPL1 enzyme responsible for the second enzymatic reaction yielding SA has not been identified yet.

Being a defence hormone triggering huge genetic and metabolic changes, SA levels must be tightly regulated. First of all, most of SA produced is rapidly inactivated by conjugation with glucose (Dean et al., 2005). After its biosynthesis in the chloroplast, SA is converted by the cytoplasmic SA glucosyl-transferase into the biologically inactive SA-glucoside which is stored in the vacuole. Hydrolases then release the active free SA during pathogen challenge. Another level of SA regulation is via gene expression. Both ICS1 and SA glucosyl-transferase mRNA transcription levels are up-regulated during SAR (Gruner et

al., 2013). This is achieved by the interplay of transcription factors acting as activators or repressors. For example, the *ics1* promoter sequence directly interacts with SARD1 and CBP60g factors (Zhang et al., 2010). Plants lacking these factors are compromised in their ability to produce SA and lack systemic defence responses. Therefore, these proteins act as *ics1* activators. On the other hand, *ics1* gene repression prior to infection is mediated by proteins such as EIN3, EIL1 and ANAC019 (Fu and Dong, 2013). Of note, these repressors have also been implicated in JA/ET signaling against necrotrophic pathogens illustrating how cross-talk among various defence pathways can be achieved.

2.1.3.2.3 SA perception and the NPR1 regulator

Increased SA levels, being either produced in cells endogenously after signal perception or applied exogenously to plant tissue by researchers, trigger major changes in gene transcription leading to systemic defences. However, the receptor responsible for detecting SA remained unidentified for decades. Efforts to find SA-binding proteins yielded several candidates, including enzymes such as the catalase, peroxidase or glutathione S-transferase (Chen et al., 1993; Durner and Klessig, 1995, Tian et al., 2012). Due to the involvement of these enzymes in reactive oxygen metabolism and redox homeostasis, it was hypothesized that SA triggers hydrogen peroxide generation leading to *pathogenesis-related* (*PR*) gene expression (Chen et al., 1993). This idea turned out to be incorrect when a subsequent study demonstrated that hydrogen peroxide is not a second messenger acting downstream of SA (Bi et al., 1995).

Furthermore, genetic studies do not support the possibility that any of these proteins are involved in SA detection (Fu and Dong, 2013).

Recently, two independent studies were successful in finally identifying SA-binding proteins clearly implicated in SAR signaling: the non-expressor of PR genes 1 (NPR1; Wu et al., 2013) and its paralogs NPR3 and NPR4 (Fu et al., 2012). Numerous genetic screens of SA-nonresponsive mutants resulted in finding mutations in the *npr1* locus (Cao et al., 1994). The inability to discover other mutated genes suggests either that the proteins involved are essential for plants or that the pathway from SA to *PR* genes is quite short. The latter seems to be more likely due to data demonstrating that NPR1 itself is the SA receptor which then acts as a transcriptional coactivator enhancing *PR* transcription via TGA transcription factors (Wu et al., 2013; Boyle et al., 2009).

NPR1 long eluded identification as an SA receptor probably due to the lability of SA-NPR1 interaction. Commonly used solid-phase experiments were unable to detect this interaction possibly because there is quick re-equilibration of SA into the mobile phase (Wu et al., 2013). Conversely, use of an equilibrium method, such as dialysis, was successful in determining that radioactively-labeled SA binds to NPR1 with a dissociation constant comparable with those of other hormone-receptor interactions (Wu et al., 2013). The same study observed that this interaction is mediated by the transition metal copper and two cysteines ($Cys^{521,529}$) of NPR1. These cysteines were previously shown to be necessary, along with SA, to activate the SAR marker gene *PR1 in vivo* (Rochon et al., 2006).

Prior to SA presence, NPR1 was found in the form of oligomers of unknown stoichiometry which disassemble during SAR (Mou et al., 2003). The emergence of NPR1 monomers was initially attributed to the creation of reducing conditions after SA accumulation in the cell since these monomers were detected *in vitro* after treatment using an SA analogue as well as after treatment with the reducing agent dithiothreitol (DTT; Mou et al., 2003). In contrast to these results, gel filtration analyses more accurately mimicking native conditions demonstrated that NPR1 with DTT did not deoligomerize, whereas NPR1 with SA did (Wu et al., 2013). It therefore seems that reducing conditions were necessary but not sufficient for disassembly due to the need to keep $Cys^{521,529}$ reduced for proper metal binding. Furthermore, conformational change caused by SA binding to the C-terminal domain of NPR1 promoted deoligomerization by disrupting interaction between the N and C-terminal domains of NPR1 (Wu et al., 2013).

Additionally, NPR3 and NPR4 can also bind SA with varying affinities (Fu et al., 2012). They were shown to act as adaptor proteins involved in NPR1 degradation (Fu et al., 2012). More specifically, they interact with Cullin 3 ubiquitin E3 ligase which then targets NPR1 for degradation by the proteosome. NPR4 interacts with NPR1 and is responsible for its constitutive turn-over under non-induced conditions. Indeed, the *npr4* mutant contains elevated levels of NPR1 compared to wild type. Additionally, its relatively low dissociation constant means it readily interacts with SA which in turn disrupts the NPR1-NPR4 interaction and abolishes constitutive NPR1 degradation (Fu et al., 2012). On the other hand, NPR3 binds with SA with much lower affinity, as can be indicated by

a significantly higher dissociation constant relative to that of NPR4. SA binding promotes the NPR3-NPR1 interaction suggesting a possible role for NPR3 in mediating NPR1 degradation at higher SA concentrations. This effect can be observed in the *npr3* mutant exhibiting, at least in the early stages after SA treatment, elevated NPR1 levels compared to wild type (Fu et al., 2012). However, this difference is no longer observed 8 hours post SA treatment casting a shadow of doubt on this hypothesis.

Finally, a genetic screen identified Non-recognition of BTH4 (NRB4) as a mediator of SA responses (Canet et al., 2012). Similarly as *npr1* mutants, *nrb4* plants are insensitive to SA despite its over-accumulation after inoculation and display an attenuated SAR. The NRB4 protein is part of a mediator multiprotein complex functioning as a transcriptional coactivator or corepressor, depending on the protein subunits involved (Shah and Zeier, 2013). Mediator subunit 16 is also part of this complex and is an essential SAR component (Zhang et al., 2012). Plants lacking this protein produce SA but do not express *PR* genes demonstrating that this protein functions downstream of SA. Additionally, it also positively regulates NPR1 protein accumulation. However, it is unknown if the mediator complex physically interacts with SA, NPR1 or TGA transcription factors involved in SAR.

2.1.3.2.4 NPR1 interaction with TGA transcription factors

NPR1 controls the onset of SAR via interaction with transcription factors regulating *PR* genes. The *PR1* gene, a model marker gene for SAR in Arabidopsis, contains three cis-regulatory elements (Lebel et al., 1998). Identified

using linker scanning (LS) mutagenesis of the *PR1* promoter, the LS5 and LS7 regions both contain a TGACG nucleotide sequence recognized by specific transcription factors regulating *PR1* expression. Although both LS5 and LS7 contain this DNA motif, mutation of LS5 leads to increased *PR1* transcription under both uninduced and induced conditions, while mutation of LS7 has no effect on expression in uninduced tissue. Conversely, the LS7 mutation leads to a significant decrease of transcription after treatment with an SA analog (Lebel et al., 1998). Therefore, LS5 is involved in negative regulation of *PR1* at all times, while LS7 is crucial for *PR1* induction after SA treatment.

Based on the TGACG sequence first identified in the *Cauliflower mosaic virus 35S* promoter, proteins interacting with this motif in plants were coined TGA transcription factors (Katagiri et al., 1989). They all contain a basic region/leucine zipper (bZIP) domain required for DNA binding common to many transcription factors (Jakoby et al., 2002). Binding to negatively charged DNA is facilitated by the positively charged basic domains of two bZIP proteins held together by the leucine zipper dimerization motif. In Arabidopsis, three TGA clades (I-III) are associated with plant defence mechanisms, while the remaining clade (IV) is involved in flower development (Shearer et al., 2012). Members of clade III (TGA3,7) have been implicated in basal resistance (Kesarwani et la., 2007), whereas those of clade I (TGA1,4) and II (TGA2,5,6) seem to have a role in SAR (Shearer et al., 2012).

TGA1 and TGA4 do not interact with NPR1 in yeast or in plants prior to SA treatment (Despres et al., 2003). However, their interaction in plants is observed

after SA application and is conditioned by the reduced state of two TGA cysteines ($Cys^{260, 266}$). Their oxidation, which mimics their state during uninduced conditions, hampers recruitment of NPR1. On the other hand, the functionally redundant TGA2, 5 and 6 all interact with NPR1 and the lack of all three transcription factors abolishes *PR1* induction (Zhang et al., 2003). Despite the fact that NPR1 interacts differentially with all the members of the TGA family involved in plant defence, the resulting interaction always stimulates DNA binding activity of these factors (Despres et al., 2003).

Based on this observation, a model of how NPR1 regulates *PR1* through TGAs started being formed. The simple presence or absence of NPR1 in the nucleus could modulate TGA binding to DNA and thus alter *PR1* expression. Indeed, NPR1 molecules translocate into the nucleus during SAR (Kinkema et al., 2000) and their deoligomerization in the cytosol after SA production could facilitate this transport (Mou et al., 2003). Additionally, NPR1 transcription levels are also elevated during SAR (Gruner et al., 2013). Furthermore, NPR1 degradation affects plant resistance: during uninduced conditions, NPR4 limits the amount of NPR1 (Fu et al., 2012). This ensures that SAR is not triggered prematurely, since elevated NPR1 levels either due to overexpression or lack of degradation lead to enhanced disease resistance (Cao et al., 1998; Fu et al., 2012). After SA treatment, the interaction between NPR4 and NPR1 is disrupted; however, high SA levels promote NPR1 interaction with NPR3 and possibly NPR1 degradation (Fu et al., 2012). Nevertheless, the amount of NPR1 in the *npr3* and *npr3npr4* mutant tissue is nearly identical as in wild type 8 hours after

induction suggesting that NPR3 might be important only in tissue in close proximity of infection sites where SA levels are much higher than those of systemic tissue (Fu et al., 2012).

Nevertheless, the idea of concentration-dependent NPR1 regulation of *PR1* has several flaws. First of all, NPR1 is found both in the cytosol and nucleus prior to SA (Despres et al., 2000; Kinkema et al., 2000) and only the nuclear fraction has an effect on SAR (Kinkema et al., 2000). Chromatin immunoprecipitation (ChIP) experiments studying *in vivo* conditions revealed that NPR1 is physically present at the *PR1* promoter at all times (Rochon et al., 2006). Therefore, *PR1* regulation by NPR1 via TGAs is clearly more complex, even though altering this protein's levels affects SAR by inhibiting the onset of resistance before induction.

It took several studies to fully understand the role of TGA2 and its homologs in SAR. Initial experiments demonstrated that *PR1* transcription cannot be up-regulated after SA-analog treatment when these three TGA genes are knocked out indicating that they act as activators (Zhang et al., 2003). Interestingly, the same study also noted that the triple mutant has elevated *PR1* levels regardless of treatment suggesting that these TGAs act as constant repressors. In contrast, ChIP assays showed that the TGA2-*PR1* interaction is dependent on SA (Johnson et al., 2003). These results were contradicted by a subsequent study using a different TGA2 targeting antibody (Rochon et al., 2006). The last study was successful in finally understanding TGA2 function. In an *in vivo* plant transcription assay, TGA2 alone was not capable of inducing

reporter gene transcription in both SA-treated and untreated cells. However, reporter gene activation was achieved when TGA2 was coexpressed with NPR1 in treated cells. Elevated expression was not observed in uninduced cells and further analysis using plant two-hybrid assays confirmed that NPR1 and TGA2 interact very poorly prior to SA treatment. Therefore, under uninduced conditions, TGA2 acts as a repressor of *PR1* and does not interact with NPR1 despite the fact that NPR1 is present on the promoter. After SA treatment, NPR1's affinity to TGA2 increases and NPR1 thus acts as a coactivator stimulating the DNA-binding ability of TGA2 resulting in elevated *PR1* transcription.

The contact of these two proteins is facilitated by an ankyrin repeat motif found in NPR1 (Ryals et al., 1996). Its mutation results in loss of TGA2 binding thus abolishing SAR. Another protein-protein interaction motif of NPR1 is its N-terminal Broad complex, Tramtrack, and Bric a-Brac/Pox virus and Zinc finger (BTB/POZ, or simply POZ) domain (Aravind and Koonin, 1999). Apart from interaction with Cullin 3 E3 ligase leading to protein degradation (Fu et al., 2012), this domain is required for the coactivator function after SA treatment (Rochon et al., 2006). Amino acid substitution or deletions in the POZ core abolish both transactivation and gene expression (Rochon et al., 2006). Nevertheless, the POZ is not an autonomous transactivation domain but it functions by interacting with the N-terminal domain of TGA2 (Boyle et al., 2009).

TGA2's N-terminus was characterized as a repression domain (Boyle et al., 2009). Using the same *in vivo* transcription assay as Rochon et al. (2006), it was shown that a mutated TGA2 protein lacking the first 43 amino acids

(Δ43TGA2) was responsible for increased reporter gene expression clearly demonstrating that the deleted region is responsible for the TGA2 repressor function. However, it is not an autonomous repression domain since experiments using a protein containing only the first 47 amino acids of TGA2 did not result in lower expression levels.

To better understand the molecular aspects of this repression event, the stoichiometry of His-tagged TGA2 and its N-terminal deletion mutants was studied using size exclusion chromatography, also known as gel filtration (Boyle et al., 2009). In this method, the largest complexes elute first in the void volume, while smaller ones travel slower and thus elute in later fractions. Complex size and, therefore, protein stoichiometry can be calculated with the help of a calibration curve. Both the full length TGA2 and the Δ43 mutant lacking the repression domain were found to form a higher order complex. Additionally, electromobility shift assays (EMSAs) were used to elucidate how TGA2 interacts with DNA. Using a fluorescently labelled DNA probe containing the LS7 sequence (Lebel et al., 1998), TGA2 was found to form a single complex with DNA at lower protein:probe ratios, whereas three different complexes were observed at higher ratios (Boyle et al., 2009). These results were further confirmed using gel filtration analyses. Use of DNA and an equimolar amount of TGA2 yielded a complex of 2 protein molecules with one DNA molecule. Higher TGA2 amounts also gave rise to this complex, as well as to two others (4 TGA2 with 2 DNA molecules and a void volume fraction of unquantifiable nTGA2 and

nDNA molecules). Conversely, Δ43TGA2 was capable of forming only the smallest complex with DNA (2:1) regardless of the protein:probe ratio.

Subsequent *in vivo* analysis demonstrated that TGA2 oligomers are present on the promoter prior to SA treatment but not after it (Boyle et al., 2009). Therefore, the formation of oligomeric TGA2 complexes on DNA prior to SA induction is important for *PR1* repression.

The next question to be addressed was how exactly TGA2 switches from a repressor to an activator. Previously, the *in vivo* transcription assay demonstrated that the presence of NPR1 is essential for this process (Rochon et al., 2003). The same method using both TGA2 and NPR1 deletion mutants revealed that it is the POZ domain of NPR1 which is responsible for interaction with the TGA2 N-terminus negating its repression effect (Boyle et al., 2009). Interestingly, if the repression domain is not present, then the POZ is not necessary for *PR1* activation. Further size exclusion experiments revealed that a TGA2-NPR1-DNA complex is formed *in vitro* with a ratio of 2:1:1 or 2:2:1.

Therefore, *PR1* activation is achieved by the disassembly of TGA2 oligomers on the promoter, TGA2 interaction with NPR1 via the ankyrin repeat motif and negation of TGA2 N-terminal repression capabilities by the POZ domain. The two proteins form a small activation complex triggering *PR1* transcription and SAR. Although the role of the *PR1*-encoded protein remains unknown, many *PR* genes give rise to proteins involved in defence pathways or in direct production of antimicrobial compounds (Gruner et al., 2013).

2.2 Protein kinase CK2

Cells of all organisms exert substantial energy to coordinate and tightly regulate their internal physiological processes. A key mechanism utilized for controlling various pathways is phosphorylation or dephosphorylation of the proteins involved. The addition or detachment of a phosphate group often changes protein conformation resulting in activity alteration. This simple mechanism is exploited in practically all regulatory events at the molecular level in cells.

In 1979, Hathaway and Traugh succeeded in isolating two distinct casein kinases using diethylaminoethyl-cellulose. Based on the chromatography profile, they were designed casein kinase 1 and 2 (CK1 and CK2). However, it seems that casein is in fact not physiologically phosphorylated by CK2, thus the name protein kinase CK2 was recommended to prevent possible confusion (Allende and Allende, 1995). This enzyme belongs to the protein kinase CMGC subfamily which contains all the Cyclin-dependant kinases (CDKs), Mitogen-activated protein kinases, Glycogen synthase kinases and CDK-like kinases.

It is known that protein kinase CK2 is a ubiquitous and pleiotrophic enzyme with over 300 reported substrates (Meggio and Pinna, 2003). Its function is to transport a phosphate group from either ATP or GTP to a seryl or threonyl amino acid residue of a target protein (Hathaway and Traugh, 1982). Interestingly, proteins with tyrosines affected by this kinase have also been reported (Donella-Deana, et al., 2001). Studies have shown that CK2 preferentially phosphorylates these residues if they are in the vicinity of acidic

amino acids (Meggio et al., 1994). The enormous number of target proteins underlines its role in various cellular processes. This enzyme has been shown to affect a wide array of targets involved in gene expression, signaling, cell proliferation and development (Guerra and Issinger, 1999). Recently, CK2 has been reported as a key player in cancer biology (Trembley et al., 2009). Due to its association with growth and proliferation, elevated levels of CK2 have been observed both in normal and cancer cells. However, it also acts as a potent suppressor of apoptosis, a key attribute of malignant cells.

2.2.1 The CK2 holoenzyme

The CK2 holoenzyme is classically a heterotetramer formed by two catalytic (α) subunits associated with two regulatory (β) subunits. In most eukaryotic organisms, there are two isoforms of the catalytic domain (CK2α and CK2α') but only one regulatory β isoform. In these organisms, the possible configurations are ααββ, αα'ββ or α'α'ββ. However, plants usually have multiple genes encoding both domains resulting in a higher number of possible configurations (Velez-Bermudez et al., 2011). This variability is made possible by the assembly process of the CK2 tetramer: in the first stage, a dimer is formed by two β domains which subsequently interacts with two α domains on either side. In the complete holoenzyme, the two catalytic domains are not in direct contact (Bibby and Litchfield, 2005).

2.2.2 The CK2α catalytic subunit

The CK2α domain is responsible for the catalysis of phosphate transfer to a target protein. It is ubiquitous in all eukaryotic cells and can be active by itself

without the assistance of the regulatory subunit (Allende and Allende, 1995). Its molecular weight is usually between 38 and 44 kDa depending on the specific isoform. It is necessary for an organism's survival as simultaneous knock-outs of genes encoding all CK2α isoforms are lethal for yeast and probably for any other organism as well (Padmanabha et al., 1990).

CK2α displays high sequence similarity among various species (Guerra and Issinger, 1999) and is closely related to the cdc2 group of protein kinases (Riera et al., 2001a). This high degree of conservation indicates that between species, even the main CK2α function may be conserved. In fact, the protein kinase CK2 contains 11 conserved sequences found throughout the protein kinase family (Hanks et al., 1988) and another three conserved regions found only in various CK2α isoforms (Challiot et al., 2000). All of these regions are found in the α domain's core which is highly conserved among species (Guerra and Issinger, 1999). This similarity is not surprising since these regions are important for the kinase function. They are vital for binding ATP (or GTP), mediating interaction with the regulatory CK2 subunit, contain phosphorylation sites of the CK2α itself and interact with the acidic region of a target protein as well as with the target seryl or threonyl (Blanquet, 2000).

The most variability among species is found in the C or N-terminal regions of CK2α. In humans, the amino acid sequence similarity of the α and α' isoforms is 75% (Guerra and Issinger, 1999). However, when the C-terminal region of α is not taken into consideration, a higher sequence homology of 86% is revealed. Interestingly, the variable terminal regions do not have a significant effect on the

enzymatic activity of the CK2α subunit, at least not under *in vitro* conditions (Guerra and Issinger, 1999). Conversely, these regions have a great effect on the overall stability of the domain. The CK2α from *Zea mays* lacks 40 C-terminal amino acids found in the human isoform resulting in higher stability sufficient for protein crystallization (Riera et al., 2001a). Moreover, in an effort to crystallize the human CK2α, a C-terminal deletion mutant had to be created to increase protein stability (Ermakova et al., 2003). Additionally, these sequence variations can possibly lead to differential localization. The α' isoform is highly and exclusively expressed in the brain and testis of mammals and, therefore, must have a specific function in these tissues. On the other hand, α is expressed ubiquitously in the body (Guerra et al., 1999).

2.2.3 Plant CK2α isoforms

A major difference between the plant CK2α and that of other eukaryotic organisms is in the number of genes encoding its various isoforms and the variability of their N-terminal sequences (Riera et al., 2001a). In total, four genes have been identified in *Arabidopsis thaliana* (Salinas et al., 2006). These genes have similar expression levels in stems, leaves, roots and inflorescences. However, one isoform containing a putative chloroplast target peptide exhibits overall increased expression. This α isoform (α4) is found only in chloroplasts, whereas the others are found predominantly in the nucleus. This localization is supported by the fact that most CK2 target proteins are either involved in signal transduction or gene expression processes occurring in the nucleus (Salinas et al., 2006).

Protein kinase CK2 has been reported to be a key player in many physiological processes in plants such as cell cycle control, growth and development, circadian rhythm, and light signaling (Mulekar et al., 2011). However, the precise effect of specific α isoforms in these processes was not studied until recently. Mulekar et al. (2011) succeeded in identifying three mutants lacking the *α1*, *α2* and *α3* subunits, respectively. Additionally, double and triple mutants were created to determine the specific effects of the various CK2 isoforms. The CK2 *α1α2α3* triple mutant is viable and exhibits approximately 70% kinase activity compared to wild type plants confirming previous findings (Salinas et al., 2006).

Various plant processes, in which CK2 has been reported to play a role, were examined using all α single, double and triple mutants by Mulekar et al. (2011). The study showed that CK2α subunits promote flowering time under both long-day and short-day conditions. Interestingly, all single mutants and double mutants flowered at the same time as wild type. Conversely, the triple mutant flowered significantly later under the same conditions and also had a higher number of rosette leaves. These discrepancies among the mutants suggest possible overlapping function and genetic redundancy of the distinct CK2α isoforms. Furthermore, the expression levels of several molecular markers for flowering time were examined. Results revealed considerable down-regulation of *FT* and *SOC1* genes (two floral integrators responsible for switching on the flowering phase) in the triple mutant under long-day conditions.

In subsequent experiments, the triple mutant displayed smaller cotyledons and reduced hypocotyls lengths under monochromatic light conditions, had a significantly reduced number of lateral roots, and showed hyposensitive responses to abscisic acid and high salt concentrations. Such significant effects were not observed in the single and double mutants. In conclusion, this study clearly demonstrated that the CK2α isoforms are important in many vital mechanisms in Arabidopsis and that their effects are additive.

2.2.4 The CK2β regulatory subunit

The regulatory subunit of protein kinase CK2 is unique and exhibits no sequence similarity to other protein regulatory domains (Riera et al., 2001a). Its molecular weight is between 26-40 kDa. The precise function of this subunit has not been fully understood but it has been shown that it confers stability and substrate specificity and modulates activity of the catalytic subunit (Blanquet, 2000). Despite the fact that CK2α functions by itself *in vitro* (Allende and Allende, 1995), physiologically the holoenzyme is in the form of a heterotetramer in most organisms.

As mentioned previously, the first step of CK2 assembly is the dimerization of two β subunits. This interaction is facilitated by a zinc-finger region containing four vital cysteine residues in each subunit (Bibby et al., 2005). Each CK2β molecule interacts with one catalytic subunit using its C-terminal positive regulatory domain. This way the CK2α is stabilized and its activity is enhanced (Chantalat et al., 1999). Other significant functional regions of the CK2β domain are the N-terminal autophosphorylation sites, amino acid

sequences probably responsible for interaction with nuclear or transmembrane proteins, a region important for proteolysis and an acidic loop responsible for CK2 down-regulation by polycationic effectors (Blanquet, 2000).

2.2.5 Plant CK2β isoforms

In contrast to most animals, plants can have multiple CK2β isoforms. While mammals have just one gene encoding the regulatory subunit, the number differs greatly among plant species. For instance, most algae have just one gene, maize and *Arabidopsis thaliana* have 4 and the apple tree (*Malus domestica*) holds the record with 11 genes encoding CK2βs (Velez-Bermudez et al., 2011). These variations raise the question if the various isoforms are genetically redundant and have overlapping functions.

The levels of sequence homology among regulatory domains of yeast, mammals and plants are not high. For example, in comparison with mammalian CK2βs, plant isoforms include an additional N-terminal extension of about 90 amino acids, lack a sequence of approximately 20 amino acids in the C-terminus and contain an altered acidic loop region (Velez-Bermudez et al., 2011). However, all plant CK2β isoforms maintain their central core containing the zinc-finger dimerization motif but have variable N and C-terminal sequences. In 2001, Riera et al. compared sequences of three (out of four) isoforms found in maize (CK2β1, β2 and β3). The results revealed that the main differences are in the N-terminal region where multiple putative phosphorylation sites are found. The presence or lack of a certain site could consequently affect the function of a specific isoform. At the amino acid level, the sequence identities between

CK2β1/β2, CK2β1/β3 and CK2β2/β3 are 77, 75 and 80%, respectively (Riera et al., 2001b).

Evidence shows that such variation changes the interaction among these subunits when forming the crucial CK2β dimer. For example, the strongest interaction is observed between CK2β1/β3 and CK2β3/β3, whereas no detectable interaction is observed between two CK2β2 molecules. All the other combinations show weak but detectable interactions. Moreover, all three β isoforms are capable of interacting with all three α isoforms examined in this study (Riera et al., 2001b), although the levels of intensity are different.

The three distinct regulatory β subunits not only have altered interaction potential, but also differential expression patterns in maize. Northern-blot analysis revealed highest expression levels of *CK2β3* in various tissues at the early stage of embryo development (1, 4 and 7 days after pollination). However, *CK2β2* is expressed the most in the intermediate stages (10 days after pollination) and *CK2β1* has its expression peak even later (Riera et al., 2001b). These results suggest that the various isoforms of the regulatory CK2 domain are not redundant. This could be true for other plant species as well since only CK2β3 and β4 are important in Arabidopsis circadian-clock regulation (Perales et al., 2006). Furthermore, experiments done on tobacco also provide evidence of differential gene expression patterns of various CK2 isoforms in the plant cell cycle (Espunya et al., 2005) and differential substrate specificity is exhibited by various CK2 holoenzymes (Dennis and Browning, 2009).

In conclusion, the protein kinase CK2 is an intriguing enzyme not only because of the amount of possible substrates and, therefore, all the processes in which it plays an important role, but also due to its versatility based on different pairings of various isoforms.

2.2.6 The role of CK2 in SAR

Rapid changes in transcription factor activity via phosphorylation or dephosphorylation have been observed in many plant signaling processes including disease resistance (Sheel, 1998). Early studies focusing on tobacco plants revealed several kinases and their possible targets involved in SAR (Sheel, 1998). For example, DNA-binding activity of protein factors termed SA response factor (SARF) and Activation sequence factor 1 (ASF1) are enhanced by SA (Johnson et al., 2003). Subsequent analysis found that these factors are actually complexes containing, among other proteins, tobacco TGA transcription factors TGA2.2 and TGA2.1, and that alteration of affinity to DNA is caused by phosphorylation of these factors (Niggeweg et al., 2000). In a search for the responsible enzyme, experiments using kinase inhibitors singled out CK2 (Stange et al., 1997). Additionally, this study also observed increases of nuclear CK2 activity after SA treatment and suppression of defence gene expression in the presence of quercetin, a CK2 inhibitor. Finally, another cell permeable CK2 inhibitor was capable of impairing the activation effect of SA on defence gene transcription *in vivo* (Hildago et al., 2001).

Several TGA factors were produced in rabbit reticulocytes in an important study focussing on Arabidopsis protein phosphorylation (Kang and Klessig,

2005). Addition of radioactive ATP substrate showed TGA phosphorylation by endogenous protein kinases, namely of TGA2, 5, 6 and, to a lesser extent, TGA3. The remainder (TGA1,4) were not affected. Since TGA2 was substantially phosphorylated, the remainder of experiments were done on this protein.

To determine which amino acids are phosphorylated, several N-terminal and C-terminal TGA2 deletion mutants were constructed. While the C-terminal deletions had no effect on phosphorylation, the others exhibited significantly decreased incorporation of radioactive phosphate. It was therefore concluded that the kinase targets are in the N-terminus of TGA2, most probably in the first 20 amino acids which include two serines and four threonines. Further analyses using alanine substitutions of 3 candidate targets at once narrowed down the list to Ser^{11}, Thr^{12} and Thr^{16}. Decreased incorporation of radioactive phosphate into a mutant with these residues substituted by alanines was also observed when using human CK2 and Arabidopsis leaf extracts.

Finally, this study also observed that CK2 activity increases as soon as 10 minutes after SA treatment in Arabidopsis. Electromobility shift assays (EMSAs) showed a decrease in TGA2 binding to DNA after CK2 treatment. Despite this effect, the nuclear localisation of TGA2 remains unchanged (Kang and Klessig, 2005). In conclusion, this study clearly linked protein kinase CK2 to molecular components of SAR, however, the significance of this phosphorylation event within SAR remained to be elucidated.

CHAPTER 3 - MATERIALS AND METHODS MATERIALS

3.1 Materials

3.1.1 Chemicals

All consumables were purchased from Sigma (St. Louis, MO) unless stated otherwise.

3.1.2 Plasmids

PET41a (Merck Millipore, Darmstadt, Germany) plasmids containing the particular DNA construct were used for bacterial transformation. All plasmids confer antibiotic resistance to kanamycin (Km). Plasmids were stored at -20ºC.

3.1.3 Bacterial strains

All experiments used *E. coli* BL21 strain (DE3) electrocompetent cells for transformation. The only exception is the case of recombinant CK2α1 which was transformed into BL21 Rosetta ^{TM} 2 (DE3) (Merck Millipore, Darmstadt, Germany) electrocompetent cells for increased protein yields. Cells were stored at -80ºC.

3.1.4 Antibodies

The following antibodies were used for protein detection after western blot or for the chromatin immunoprecipitation procedure:

Casein kinase IIa (H-286) antibody (rabbit polyclonal IgG; Santa Cruz Biotechnology, Dallas, TX)

His-tag antibody (rabbit polyclonal IgG; GenScript USA Inc., Piscataway, NJ)

His-probe (H3) antibody (mouse monoclonal IgG; Santa Cruz Biotechnology, Dallas, TX)

NWSHPQFEK anti-Strep-tag antibody (rabbit polyclonal IgG; GenScript USA Inc., Piscataway, NJ)

Goat anti-rabbit IR Dye® 680LT (goat IgG; LI-COR, Lincoln, NE)

Goat anti-mouse IR Dye® 800LT (goat IgG; Rockland Immunochemicals Inc., Boyertown, PA)

3.2 Methods

3.2.1 Bacterial cell transformation

25µL of electrocompetent cell suspension were added to 50 fmols of plasmid DNA and MQ H_2O to a total volume of 50µL. This mixture was transferred to a chilled 0.2 gap electroporation cuvette and inserted into the Gene Pulsar electroporator (BioRad, Hercules, CA) set to 25µFD capacitance, 200Ω resistance and 2.5kV potential. Transformed cells were added to 0.5mL of 2YT media (16g/L Tryptone, 10g/L yeast extract and 5g/L NaCl) with 1M glucose and incubated at 37ºC for 1 hour shaking at 250 rpm. Cells were then plated onto selective media (2YT + 15g/L agar) containing Km (kanamycin; 50mg/L) for the BL21 strain (DE3), or Km (50mg/L) and Cm (chloramphenicol; 34mg/L) for the BL21 Rosetta ^{TM} 2 (DE3).

3.2.2 Protein production

Transformed bacterial cells obtained from a single colony were used to inoculate 5mL of 2YT media (16g/L Tryptone, 10g/L yeast extract and 5g/L NaCl) containing the appropriate antibiotics. Shaken at 250 rpm overnight at 37ºC, the 5mL saturated cell suspension was added to 0.5L of autoclaved 2YT containing the appropriate antibiotics. Cells were allowed to grow at 37ºC and 250 rpm until OD600 reached 0.4-0.6 after which 0.5M isopropyl-β-D-thiogalactopyranoside (IPTG; BioShop, Burlington, ON) was added to induce recombinant protein production. Cells were grown for another 4 hours at 37ºC and 250 rpm and harvested by centrifugation at 8000 rpm for 5 minutes (Sorvall-RC-5C-Plus; Mandel Scientific Company, Guelph, ON). The pellet was stored at -20ºC until needed.

3.2.3 Protein purification using affinity chromatography

The bacterial cell pellet was resuspended in 15mL of HisTrap binding buffer (50mM HEPES, 300mM NaCl, 40mM imidazole, pH 7.5) or Strep-Tactin binding buffer (50mM phosphate, 300mM NaCl, pH 8). Cells were disrupted using the EmulsiFlex-C3 homogenizer (Avestin, Ottawa, ON) for 3.5 min using 1700PSI pressure. Cellular debris was pelleted by centrifugation at 16200 rpm, 30 min, 4ºC (Centrifuge 5417R; Eppendorf, Hamburg, Germany). The supernatant was filtered using glass wool and subsequently filter-sterilized by PVDF syringe filters (Mandel Scientific Company, Guelph, ON) and kept on ice.

His-tagged proteins were purified using HisTrap HP 1mL columns (GE Healthcare Bio-Sciences, Baie d'Urfé, QC), while Strep-tagged proteins were purified using the Strep-Tactin 1mL column (Qiagen, Limburg, Netherlands). These columns were adapted to the AKTA purifier^{TM} 10 FPLC (GE Healthcare Bio-Sciences, Baie d'Urfé, QC) operated by the Unicorn Version 5.31 software.

The total filtered lysate was loaded onto the appropriate column followed by a washing step using 10mL of binding buffer (50mM HEPES, 300mM NaCl, 40mM imidazole, pH 7.5 for HisTrap; 50mM phosphate, 300mM NaCl, pH 8 for Strep-Tactin). Proteins were eluted using the appropriate elution buffer (50mM HEPES, 300mM NaCl, 1M imidazole, pH 7.5 for HisTrap; 50mM phosphate, 300mM NaCl, 2.5mM desthiobiotin, pH 8 for Strep-Tactin) and collected in 0.5mL fractions. Total protein amount in peak fractions was determined using the BioRad protein assay reagent (BioRad, Hercules, CA).

3.2.4 CK2 kinase treatment

Phosphorylation using the human recombinant CK2 holoenzyme (New England Biolabs Inc., Ipswich, MA) was performed according to manufacturer's instructions in 1X CK2 reaction buffer (20mM Tris-Cl, 50mM KCl, 10mM $MgCl_2$, pH 7.5) supplemented with 200µM ATP at 30ºC. 100pmols of TGA2-derived proteins were incubated with 250U (0.5µL) of CK2 for 30 minutes for western blot and EMSA analyses, 5000pmols of TGA2 proteins were incubated with 2500U (5µL) of CK2 for 3 hours for gel filtration analyses.

Phosphorylation using the affinity purified CK2α1 was performed in 20mM Tris-Cl, pH 7, 130mM NaCl, 10mM $MgCl_2$ and 200μM ATP accompanied by a prayer. 2pmols of TGA2 was incubated with 1pmol of affinity-purified CK2α1 for 30 minutes at room temperature.

3.2.5 Electromobility shift assay

The fluorescent *LS7* DNA probe used in all experiments contains a single TGA binding site and was prepared by annealing two DNA oligonucleotides:
5'-TATTTTACTTACGTCATAGATGTGGCGGCA-3'
5'-TGCCGCCACATCTATGACGTAAGTAAAATA-3'.

Annealing was accomplished by mixing equal amounts of each oligonucleotide in TEN buffer (10mM Tris-Cl, pH 7.5, 1mM EDTA and 150mM NaCl). This mixture was incubated in water at a temperature over 95ºC and let to gradually cool down overnight in the dark. Both DNA strands were labeled with IRDye® 680LT (LI-COR, Lincoln, NE) on their 5' end enabling fluorescent detection of this probe.

Binding reactions of protein and DNA were performed by mixing 2pmol of TGA2-derived protein with 100fmol of *LS7* probe in a total volume of 50μL in EMSA buffer (20mM Tris-Cl, pH 7.9, 250mM NaCl, 5% glycerol, 0.5% Tween 20 and 0.1mM DTT) and incubating at room temperature in the dark for 20 minutes. When testing the effect of another protein on this interaction (POZ + phosphorylated TGA2 + DNA), 5pmol of POZ was incubated with 2pmol of phosphorylated TGA2 and 100fmol of probe maintaining the same conditions.

After this incubation, samples were loaded onto a 4% polyacrylamide gel (29.2:0.8 acrylamide-bisacrylamide in 100mM Tris, 100mM borate and 10mM EDTA) and the electrophoresis was run at 130V/cm for 80 minutes in the dark. The gel was scanned on the Odyssey infrared scanner (LI-COR, Lincoln, NE) detecting the fluorescence of the *LS7* probe. Intensity values of detected bands were obtained using the LI-COR Image Studio software (Version 2.1.10).

3.2.6 SDS-PAGE electrophoresis

Protein samples up to a total volume of 0.5mL were precipitated by adding 1mL of 100% acetone and left at -20ºC for at least 1 hour. Proteins were then pelleted by centrifugation at 16200 rpm, 30 min, 4ºC (Centrifuge 5417R; Eppendorf, Hamburg, Germany). The pellet was resuspended in 1X SDS loading buffer (60mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 0.025% bromophenol blue and 5% mercaptoethanol) and boiled for 5 minutes. Samples were then electrophoresed using SDS-PAGE in a 12% resolving gel (29.2:0.8 acrylamide-bisacrylamide in 0.375M Tris, pH 8.8, 0.1% SDS, 0.0625% APS and 0.1% TEMED).

3.2.7 Western blot

Proteins separated by SDS-PAGE were transferred to a 0.45µm nitrocellulose membrane (BioRad, Hercules, CA) soaked in Transfer buffer (25mM Tris, 192mM glycine, 20% methanol, pH 8.3) using the Trans-Blot® SD semi-dry transfer cell (BioRad, Hercules, CA) linked to a power source set to 20V, 100mA, 5W for 75 minutes. The membrane was saturated using a blocking solution (30g milk powder/1L of TBS made of 20mM Tris-Cl, pH 7.6, and 0.137M

NaCl) for 1 hour after which it was exposed to a primary antibody diluted 1/2000 in 10g/L milk protein in TTBS (TBS + 0.1% Tween 20) for another hour. The membrane was then washed 3X using TTBS for 3 X 5 minutes and incubated with a secondary antibody diluted 1/15000 in TTBS for 1 hour in the dark as both secondary antibodies used in our experiments were fluorophore-labelled (LI-COR, Lincoln, NE). The membrane was washed 3X with TTBS and 2X with TBS for 5 X 5 minutes and scanned using the Odyssey infrared scanner (LI-COR, Lincoln, NE) detecting the fluorescence of the secondary antibody.

3.2.8 Gel filtration assay

His-tagged purified protein samples treated with or without CK2 were diluted to a final volume of 2mL using the S300 running buffer (50mM HEPES, pH 7.5, 300mM NaCl). Gel filtration analysis was performed on Sephacryl S300 HR resin packed in a 50cm long HR 16 column (GE Healthcare Bio-Sciences, Baie d'Urfé, QC) providing a column volume of 104.5mL equilibrated with S300 running buffer. This column was adapted to the AKTA purifier^{TM} 10 FPLC (GE Healthcare Bio-Sciences, Baie d'Urfé, QC) operated by the Unicorn Version 5.31 software. The samples were loaded into a 2mL injection loop and loaded on the column at a flow rate of 0.2mL/min. This rate was maintained during analysis. 0.5mL fractions were collected starting at 0.2 column volumes.

Proteins in fractions of interest were acetone precipitated and prepared for SDS-PAGE analysis as described above. Samples from 3 subsequent fractions were pooled together to improve detection and analysed via SDS-PAGE and western blot.

3.2.9 Mass analysis sample preparation

His-tagged TGA2 was purified using a HisTrap HP 1mL column (GE Healthcare Bio-Sciences, Baie d'Urfé, QC) as described above. 5000pmol of TGA2 was incubated with 2500U (5µL) of CK2 for 3 hours as described above. This was done in triplicate. The 3 samples were acetone precipitated and centrifuged as described above. The protein pellets were resuspended in 1X SDS loading buffer and the proteins separated by SDS-PAGE in a 12% gel as described above. This gel was stained for 15 minutes using the staining solution (0.1% Coomassie blue R-250, 50% methanol and 10% acetic acid) for 15 minutes. Subsequently, the gel was destained using the destaining solution (50% methanol and 10% acetic acid) to visualize protein bands and clear the background. The hearts of the bands corresponding to phosphorylated TGA2 were tightly excised and transferred to a 1.5mL MC tube. Another gel slice containing only background and no detected proteins was also excised and transferred to another 1.5mL MC tube. All these slices were then washed 2X with 1mL of 50% acetonitrile in MQ water for 2 minutes. The liquid was discarded and the samples in the tubes frozen at -80ºC and stored until they were shipped to the Harvard Microchemistry facility (52 Oxford St., NW Labs B247, Cambridge, MA) for mass spectrometry analysis.

3.2.10 Pull-down assay

Bacterial pellets containing His-tagged proteins and Strep-tagged CK2α1 were resuspended using only the HisTrap binding buffer. The bacterial lysates were prepared as described above. The lysate containing the particular

His-tagged protein was loaded onto a HisTrap HP 1mL column (GE Healthcare Bio-Sciences, Baie d'Urfé, QC) adapted to the AKTA purifier^{TM} 10 FPLC (GE Healthcare Bio-Sciences, Baie d'Urfé, QC) and operated by the Unicorn Version 5.31 software. However, lower amounts of lysates were used compared to purification procedures: 0.5mL of TGA2 lysate (from a pellet of gained from a 0.25L culture exposed to IPTG for 2 hours and resuspended in 12.5mL of HisTrap binding buffer), 3mL of Δ43TGA2 lysate (from a pellet of gained from a 0.25L culture exposed to IPTG for 4 hours and resuspended in 12.5mL of HisTrap binding buffer) and 1mL of NPR1 lysate (from a pellet of gained from a 0.25L culture exposed to IPTG for 2 hours and resuspended in 12.5mL of HisTrap binding buffer). In the case when no His-tagged protein was used, this step and the subsequent washing step were omitted.

These proteins were bound to the resin of the column and washed with 10mL of HisTrap binding buffer to remove unbound proteins. Next, a bacterial lysate containing Strep-tagged CK2α1 (from a pellet gained from 2.5L of cell culture exposed to IPTG for 4 hours and resuspended in 25mL of HisTrap binding buffer) was loaded onto the same column. Once again, the column washed with 10mL of HisTrap binding buffer to remove unbound proteins. All proteins were eluted using HisTrap elution buffer and 0.5mL fractions were collected. Peak fractions (monitored using absorbance at 280nm) were prepared for analysis using SDS-PAGE and western blot as described above.

3.2.11 Autophosphorylation assay

Experiments during which autophosphorylation of CK2α was observed were performed as follows. Bacterial cells from 2.5L of 2YT media producing the Strep-tagged CK2α were disrupted as described previously. The lysate was loaded onto the Strep-Tactin 1mL column (Qiagen, Limburg, Netherlands) adapted to the AKTA purifier^{TM} 10 FPLC (GE Healthcare Bio-Sciences, Baie d'Urfé, QC) and operated by the Unicorn Version 5.31 software. The column was first washed using 10mL of Strep-Tactin binding buffer and then using 3mL of 20mM phosphate buffer, pH 7, 130mM NaCl and 10mM $MgCl_2$. The column-bound proteins, along with 1000pmols of TGA2his, were then incubated with 20mM phosphate buffer, pH 7, containing 130mM NaCl, 10mM $MgCl_2$, 1mM DTT and 0.2mM ATP for 30 or 60 minutes. The column was then washed using 10mL of Strep-Tactin binding buffer and the proteins were eluted using the Strep-Tactin elution buffer. Peak fractions were acetone precipitated and analyzed using SDS-PAGE and Western blot as described previously.

3.2.12 Chromatin immunoprecipitation (ChIP) assay

ChIP experiments were performed as described by Chakravarthy et al. (2003). Briefly, 2g of *Arabidopsis thaliana* leaf tissue from 6 week old plants were submerged in a 1% formaldehyde solution and stirred for 30 minutes at room temperature. The fixed tissue was repeatedly washed (3X) with distilled water, dried and stored at -80ºC. Plant tissue was ground with 2ml of IP buffer (50mM Tris, pH 7.9, 150mM NaCl, 0.1% SDS, 1% Triton X-100 and 1mM EDTA) and 1X Protease inhibitor cocktail. Leaf debris was pelleted by centrifugation at 16200

rpm, 5 min, 4ºC (Centrifuge 5417R; Eppendorf, Hamburg, Germany) and the supernatant was sonicated (Sonic Dismembrator Model 100; Fisher Scientific, Ottawa, ON) at level 10 for 6 × 20 seconds of continuous blasts with 40 second rests on ice in between each blast. This was done to shear genomic DNA fixed to interacting proteins. This lysate was further centrifuged for 5 minutes and filtered using a PVDF syringe filter (Mandel Scientific Company, Guelph, ON) to remove additional plant debris.

Protein A agarose beads (50µL; Affi-gel protein A Gel; BioRad, Hercules, CA) were washed once with 0.5mL IP buffer and incubated with 10µL of antibody (anti-CKIIa; Santa Cruz Biotechnology, Dallas, TX) diluted in 0.5mL of IP buffer for 1 hour on a rotating wheel (Roto-torque; Colepalmer instrument company, Montreal, QC). Beads were pelleted by quick centrifugation (5 sec) and the supernatant was removed. The beads with the affixed antibody were washed 3X with IP buffer and incubated with the sonicated leaf lysate overnight at 4ºC on the rotating wheel. Beads were then washed 3X with IP buffer and 2X with TE buffer (100mM Tris and 50mM EDTA). The formaldehyde crosslinking was reversed by incubation of beads with TE buffer + 1% SDS for 15 minutes at 65ºC. The beads were discarded and the supernatant was further incubated at the same temperature overnight.

DNA contained in the samples was isolated using two precipitation procedures using sodium acetate and isopropanol each followed by centrifugation at 16200 rpm, 30 min, 4ºC (Centrifuge 5417R; Eppendorf, Hamburg, Germany). The precipitated DNA was resuspended in 20µL of TE

buffer and 4µL of this solution were subjected to 10 cycles of polymerase chain reaction (PCR) to amplify the isolated DNA sequences using specific PCR primers (total reaction volume 25µL). The PCR product (5µL) was further amplified and analysed using quantitative PCR (qPCR) reactions in a total volume of 20µL using the BioRad iQ SYBR Green Supermix kit according to manufacturer's instructions (BioRad, Hercules, CA). Forty cycles of a two temperature protocol were used.

Three biological replicates were performed from each tissue. Recorded CT values obtained using the tested primer pairs were compared with the CT value of the UBQ5 primer pair. The final values are the relative:

$2^{(\text{CT tested primer pair} - \text{CT UBQ5})}$.

The PCR primers used in this study were:

$UBQ5_F$	5'-GACGCTTCATCTCGTCC-3'
$UBQ5_R$	5'-GTAAACGTAGGTGAGTCCA-3'
$PR1\text{-}1_F$	5'-CTAAAACCCCAAGCATTTG-3'
$PR1\text{-}1_R$	5'-TGTGTACCTGGTACATAAAT-3'
$PR1\text{-}2_F$	5'-GCGGTGTAGATACTGCATTC-3'
$PR1\text{-}2_R$	5'-ATGTTACAGTTAGCCACTAG-3'

CHAPTER 4 - RESULTS - TGA2 PHOSPHORYLATION BY CK2

4.1 DNA binding capabilities of TGA2 mutants lacking the putative phosphorylation site are affected by protein kinase CK2

Phosphorylation of the Arabidopsis transcription factor TGA2 by protein kinase CK2 was initially investigated by Kang and Klessig (2005). In this study, CK2 activity gained from rabbit reticulocytes, Arabidopsis leaf extracts as well as recombinant human CK2 was used to determine target phophorylation sites in TGA2. Based on these experiments, the authors concluded that the putative phosphorylation motif is found among the first 20 amino acids of the N-terminus. More specifically, one serine and two threonines (Ser^{11}, Thr^{12} and Thr^{16}) were found to be critical for efficient phosphorylation (Kang and Klessig, 2005).

To further investigate this event at the molecular level and its role in SAR, we generated TGA2 mutants lacking this putative motif and containing an additional C-terminal His tag. As seen in Figure 1A, the three residues deemed critical by the previous study were substituted either by aspartates (TGA2-3D) or alanines (TGA2-3A). Aspartate point mutations are often used in phosphorylation studies to mimic phosphorylated residues due to the constitutive negative charge of aspartate (Tyler et al., 2009; Miyoshi et al., 2006). Conversely, substitutions with unphosphorylatable alanine give rise to mutants not affected by kinases thus representing constitutively nonphosphorylated proteins.

We produced all three proteins in *E. coli* and purified them via affinity chromatography by using HisTrap™ HP columns which bind His-tagged proteins.

100 pmols of each purified protein were incubated with recombinant human CK2 (New England Biolabs). The reaction was stopped by precipitating the proteins in acetone at -20ºC. Afterwards, proteins in these samples were separated by sodium dodecyl sulphate-containing polyacrylamide gel electrophoresis (SDS-PAGE) and immunobloted. His-tagged proteins transferred on the membrane were detected using a primary anti-His-tag antibody along with a fluorophore-coupled secondary antibody. This method was successful in detecting TGA2 phosphorylation due to the fact that there is an identifiable shift in migration of the phosphorylated protein compared to the unphosphorylated form (Figure 1B). Slower migration of phosphorylated proteins is a common observation (Wegener and Jones, 1984; Peck, 2006). It is partially caused by the increase of molecular weight by the addition of phosphate groups but mainly due to additional negative charges influencing the interaction of phosphorylated proteins with negatively charged SDS molecules (Peck, 2006). Unexpectedly, there was also a slight change of migration of the phosphorylated forms of TGA2-3D, although this effect was not as pronounced as in the case of the wild type protein (Figure 1B). The migration of TGA2-3A in SDS-PAGE seemed unaffected by phosphorylation.

Electromobility shift assays (EMSAs) using a fluorescently labelled DNA probe containing the *LS7* binding site, which contains a single TGA2 binding sequence (Lebel et al., 1998), previously revealed that TGA2 can form several different complexes on DNA depending on the protein:DNA probe ratio (Boyle et al., 2009). At a 20:1 ratio, TGA2 forms a dimer on one molecule of the *LS7* DNA probe which is observed in EMSA as a single retarded band. This protein:probe

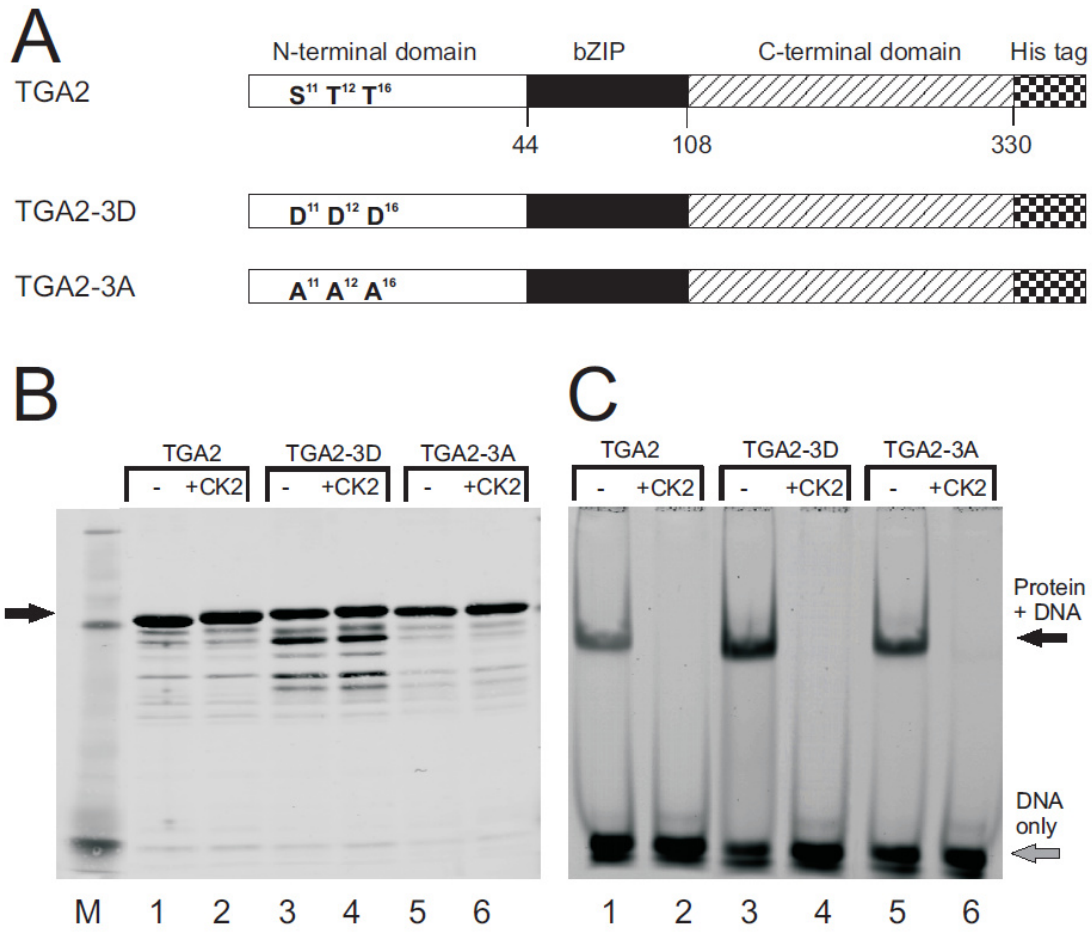

Figure 1. TGA2 and its substitution mutants are phosphorylated by human recombinant CK2.

(A) Schematic representation of proteins tested for CK2 phosphorylation in **(B)** and **(C)**, namely the His-tagged TGA2 and its mutants TGA2-3D and TGA2-3A in which the putative phosphorylation motif (S^{11}, T^{12}, T^{16}) is substituted by aspartates (D) or alanines (A), respectively. TGA2's N-terminal domain, basic domain/leucine zipper (bZIP) and C-terminal domain, as well as the His tag, are also depicted along with the numbers corresponding to starting amino acids of these domains.

(B) Immunoblot analysis of TGA2, TGA2-3D and TGA2-3A proteins incubated with (+CK2) or without (-) human recombinant CK2. These proteins were produced in *E. coli*, HisTrap purified and incubated with or without 250 U of CK2

for 3 hours at 30ºC. The protein samples were acetone precipitated, separated using 12% SDS-PAGE and transferred to a nitrocellulose membrane. Proteins of interest were detected via a primary anti-His-tag antibody and a secondary fluorophore-coupled antibody using the Odyssey imager. Lanes 1 and 2 illustrate the slight difference in migration of unphosphorylated and phosphorylated TGA2, respectively. Migration changes of TGA2-3D (lanes 3 and 4) and TGA2-3A (lanes 5 and 6) are not as apparent. A black arrow points to the full length proteins used in this experiment. Smaller bands detected in all lanes are most likely degradation products cleaved by bacterial proteases during production. Lane M contains the protein standard marker.

(C) Electromobility shift assay using TGA2, TGA2-3D and TGA2-3A proteins incubated with (+CK2) or without (-) human recombinant CK2. 1 pmol fractions of protein samples from **(B)** that were produced in *E. coli*, HisTrap purified and incubated with or without 250 U of CK2 for 3 hours at 30ºC, were additionally incubated with the fluorophore-coupled *LS7* DNA probe in EMSA buffer for 20 minutes in the dark. These were subsequently electrophoresed for 80 minutes at 130 V in a 4 % EMSA gel. The fluorescent DNA probe was detected by the Odyssey imager. The DNA probe not interacting with any proteins was detected at the bottom of the gel (grey arrow), while the DNA-protein complex displayed decreased mobility (black arrow). Clearly, all 3 analysed proteins bind to DNA (lanes 1, 3 and 5), however, their phosphorylated forms do not (lanes 2, 4 and 6).

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ratio was maintained in our experiments. As can be seen in Figure 1C, the TGA2-DNA complex yielded a single retarded band (black arrow), while the probe not interacting with any protein migrated further down the gel (grey arrow). Phosphorylation by CK2 negatively affected the DNA-binding capabilities of TGA2 and, therefore, a retarded band was no longer detected (Figure 1C, lane 2). Surprisingly, an identical effect was observed when the TGA2 mutants were

tested. DNA binding of both these mutants was abolished after their incubation with CK2 (Figure 1C, lanes 4 and 6). Despite the fact that an identical mutant as our TGA2-3A was previously reported to be minimally phosphorylated by recombinant human CK2 when compared to TGA2 (Kang and Klessig, 2005), our results clearly show that both the 3A and 3D mutants are phosphorylated. Of note, the phosphomimetic TGA2-3D is still capable of binding to DNA prior to kinase treatment, a second result refuting the previously published hypothesis that only Ser^{11}, Thr^{12} and Thr^{16} form the putative phosphorylation motif affecting binding to DNA (Kang and Klessig, 2005). In conclusion, both the immunoblot and the EMSA data suggest the possibility of additional CK2 target sites found in TGA2.

4.2 Protein complex deoligomerization is observed after phosphorylation or when the putative phosphorylation site is mutated

A previous study focusing on TGA2's molecular characteristics used size exclusion chromatography to analyze stoichiometry of complexes formed by this transcription factor (Boyle et al., 2009). It was found that full length TGA2 forms higher-order oligomeric complexes containing more than 40 protein molecules. On the other hand, N-terminal deletion of the first 93 amino acids resulted in formation of protein dimers and monomers. Conversely, shorter N-terminal deletion mutants remained oligomeric so it was concluded that it is the leucine zipper motif (residues 72-86) that is necessary for oligomerization. Additionally*, in vivo* data from the same study show that high-order TGA2 complexes are present at the *PR1* promoter prior to SA application and disappear after SA treatment

(Boyle et al., 2009). Furthermore, SA treatment leads to rapid increases of protein kinase CK2 activity (Kang and Klessig, 2005). For these reasons we hypothesized that CK2 phosphorylation could result in rearrangement of TGA2 complexes, a possible requirement for subsequent *PR1* gene activation.

To test our hypothesis, we chose to analyze protein complex stoichiometry via the same method as Boyle et al., (2009): size exclusion chromatography. Briefly, we purified recombinant His-tagged TGA2 and its mutants (TGA2-3D and TGA2-3A) using affinity chromatography with subsequent analysis via Sephacryl S300 gel filtration. TGA2 phosphorylation by CK2 (when indicated) was performed prior to this analysis. In theory, gel filtration can separate proteins or their complexes up to the column's size exclusion limit. Protein complexes larger than this limit will elute earliest in the void volume fractions. Smaller complexes or molecules will elute later in the included volume fractions. The size of protein complexes or monomeric proteins eluted between the exclusion limit and inclusion limit (at approximately one column volume) and, therefore, within the included volume, is logarithmically proportional to their respective retention volume. A calibration curve using monomeric protein standards of known molecular weights is needed to precisely describe the mathematical relationship and is used to calculate the size of a protein complex of interest based on its elution volume (see Supplemental Figure 1).

Also shown by Boyle et al. (2009), we confirmed that nonphosphorylated TGA2 formed oligomers of unknown stoichiometry (Figure 2A). This can be seen in the elution profile as most of the protein eluted in the void volume (~35.5ml)

indicating that the protein complexes are larger than 1.5 MDa, the theoretical Sephacryl S300 size exclusion. His-tagged TGA2 has a molecular weight of approximately 37.3 kDa and, therefore, the eluted complexes are formed by 40 or more TGA2 molecules. This result was confirmed by immunoblot analysis using a primary anti-His-tag antibody and secondary fluorophore-coupled antibody where only TGA2 oligomers in the void volume and no smaller complexes in the subsequent fractions were identified (Figure 2A, inset). TGA2 incubated with CK2 yielded a similar elution profile, as the majority of phosphorylated TGA2 (phTGA2) was seen in the void volume. However, immunoblot analysis revealed a small portion of protein in the included volume (~69ml) which was determined to contain monomers of phTGA2 (Figure 2B, inset). This result confirms our hypothesis that phosphorylation results in partial protein complex rearrangement.

A similar deoligomerization effect was also seen with the TGA2-3D and 3A mutants. Interestingly, these proteins didn't need CK2 to deoligomerize. Although their elution profiles did not hint of any smaller complexes, more sensitive immunoblot analyses showed a broader redistribution (Figure 2C and 2D, insets). In native conditions, both proteins form mostly oligomers, but also monomers, dimers, trimers and tetramers. These results imply that multiple substitutions in the N-terminus of TGA2 have a similar effect on interprotein interactions as phosphorylation, possibly via numerous hydrogen bond alterations leading to conformational changes and partial deoligomerization.

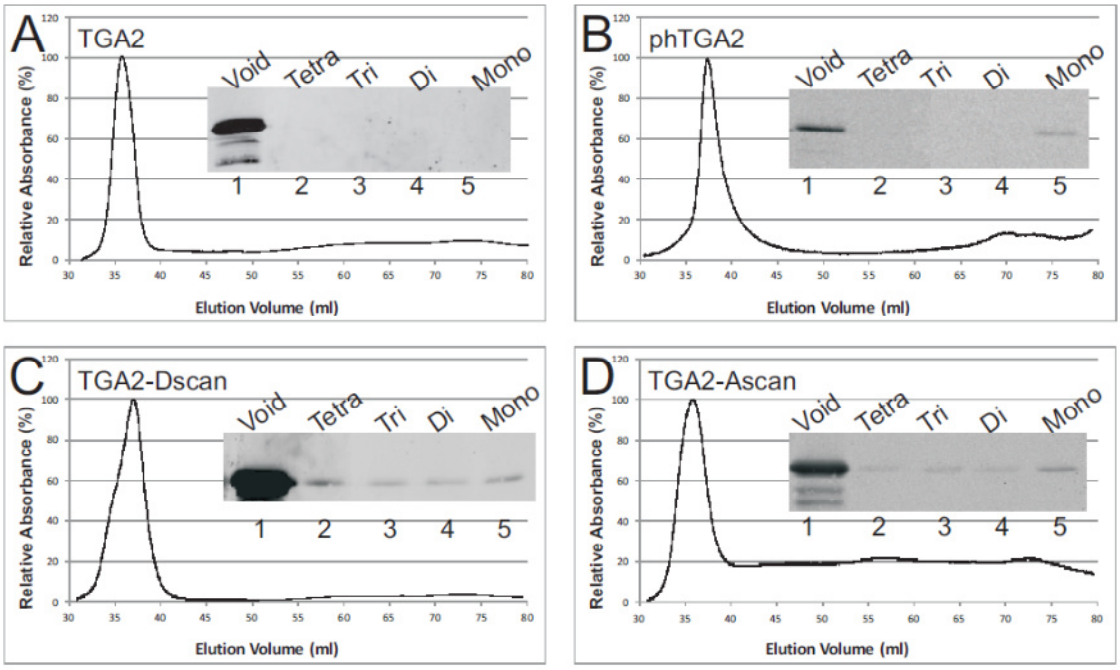

Figure 2. Deoligomerization of protein complexes due to CK2 phosphorylation or substitution mutations.

Elution profiles of *E. coli* expressed and HisTrap purified TGA2 **(A)**, TGA2 incubated with human recombinant CK2 (phTGA2) **(B)**, TGA2-3D **(C)** and TGA2-3A **(D)** proteins analysed by size exclusion chromatography and monitored by absorbance measured at 280nm. The highest absorbance value of each analysis was considered as 100% and the absorbance value measured at 30ml was arbitrarily set to 0%. Fractions corresponding to the oligomeric state found in the void volume (Lane 1, insets) along with those in the included volume corresponding to protein tetramers, trimers, dimers and monomers (Insets, lanes 2-5, respectively) were acetone precipitated, electrophoresed and transferred to a nitrocellulose membrane. Proteins of interest were detected via a primary anti-His-tag antibody and a secondary fluorophore-coupled antibody using the Odyssey imager.

4.3 The N-terminus is not required for interaction of TGA2 with CK2

In an attempt to determine which residues, in addition to Ser^{11}, Thr^{12} and Thr^{16}, are targeted by CK2, we constructed several TGA2 N-terminal deletion mutant proteins with the first 12, 24 and 43 amino acids removed (Δ12TGA2, Δ24TGA2 and Δ43TGA2, respectively; see Figure 3A). The reason why we focused on the N-terminus was not only due to the location of the previously identified phosphorylation motif, but also because of the abundance of phosphorylatable residues as well as the presence of multiple aspartates, which are often found in CK2 target motifs (Meggio et al., 1994).

All three N-terminal deletion mutants were produced in *E. coli* and affinity purified. Protein samples were incubated with or without human recombinant CK2, acetone precipitated and separated via SDS-PAGE with subsequent immunoblot analysis. Electrophoretic mobility of phosphorylated Δ12TGA2 was slightly altered, the corresponding protein band being slightly higher than that of the nonphosphorylated protein (Figure 3B, lanes 1 and 2), in a similar manner as in the case of full length TGA2 (Figure 1B, lane 2). Although protein bands of Δ24TGA2 and Δ43TGA2 exposed to CK2 did not show any mobility shift compared to the unphosphorylated protein, the shape of their corresponding band is slightly altered (Figure 3B, lanes 4 and 6). This effect cannot be attributed to usual reasons, such as higher protein amounts or varying salt and detergent levels, since the two samples (nonphosphorylated and phosphorylated) are aliquots with the only difference being a very small volume of added enzyme.

However, the change of band shape was most likely caused by phosphorylation, as this effect has been seen in other studies (Lao et al., 2007).

Furthermore, EMSA results clearly show that CK2 treatment of Δ12TGA2 and Δ24TGA2 results in total loss of DNA binding (Figure 3C, lanes 2 and 4). Conversely, interaction of Δ43TGA2 with the probe is not abolished or even slightly diminished by phosphorylation (Figure 3C, lane 6), despite the relatively long enzyme reaction time period (3 hours). These results suggest that the area responsible for regulating the affinity of TGA2 to DNA is located between amino acids 25 and 43. Of note, the Δ24TGA2 mutant forms a complex with DNA with decreased mobility compared to all other protein complexes tested thus far. It is possible that this particular mutant binds to DNA with altered stoichiometry. As the full length TGA2 forms dimers with one DNA molecule (Boyle et al., 2009), the number of molecules of this protein mutant binding to the probe is most likely higher.

Additional gel filtration and immunoblot analyses of these mutants uncovered that these proteins form only higher-order oligomers prior to phosphorylation (Figure 3D), whereas incubation in the presence of CK2 results in partial deoligomerization leading to the emergence of smaller complexes or monomers. Therefore, all of the tested N-terminal deletion mutants do in fact interact with and are subsequently phosphorylated by CK2, even though this modification doesn't seem to have an effect on the DNA-binding ability of Δ43TGA2.

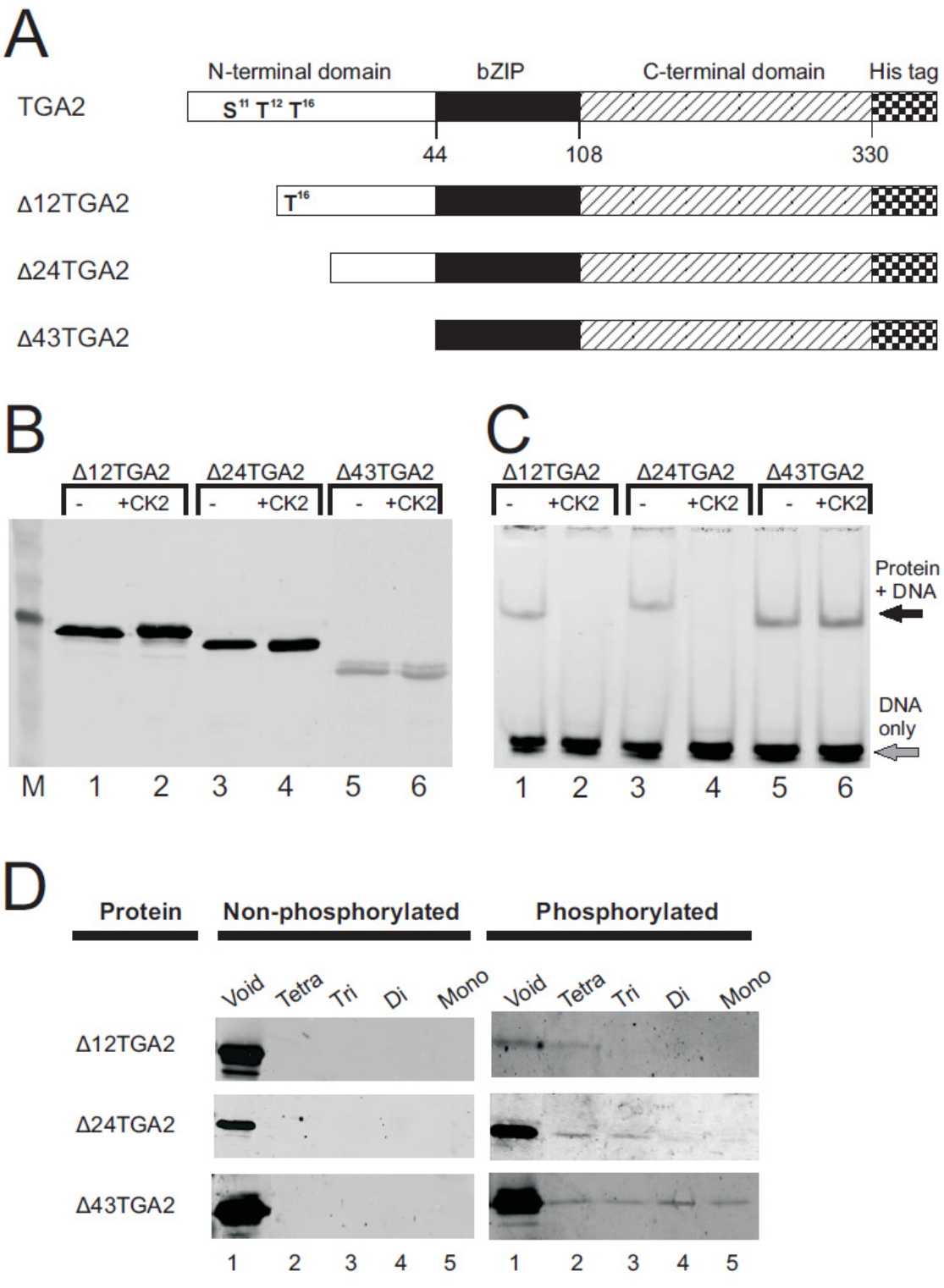

Figure 3. Effects of CK2 phosphorylation on TGA2 N-terminal deletion mutants.

(A) Schematic representation of proteins tested for CK2 phosphorylation in **(B)**, **(C)** and **(D)**, namely the His-tagged full length TGA2 containing the putative phosphorylation motif (S^{11}, T^{12}, T^{16}) and the N-terminal deletion mutants lacking the first 12, 24 and 43 amino acids (Δ12TGA2, Δ24TGA2 and Δ43TGA2, respectively). TGA2's N-terminal domain, basic domain/leucine zipper (bZIP) and C-terminal domain, as well as the His tag, are also depicted along with the numbers corresponding to starting amino acids of these domains.

(B) Immunoblot analysis of Δ12TGA2, Δ24TGA2 and Δ43TGA2 proteins incubated with (+CK2) or without (-) human recombinant CK2. These proteins were produced in *E. coli*, HisTrap purified and incubated with or without 250 U of CK2 for 3 hours at 30ºC. The protein samples were acetone precipitated, separated using 12% SDS-PAGE and transferred to a nitrocellulose membrane. Proteins of interest were detected via a primary anti-His-tag antibody and a secondary fluorophore-coupled antibody using the Odyssey imager. Slight differences in migration of unphosphorylated and phosphorylated Δ12TGA2 (Lanes 1 and 2, respectively), as well as slight shape changes of protein bands corresponding to phosphorylated Δ24TGA2 (Lanes 3 and 4, respectively) and Δ43TGA2 (Lanes 5 and 6, respectively) can be observed. Lane M contains the protein standard marker.

(C) Electromobility shift assay using Δ12TGA2, Δ24TGA2 and Δ43TGA2 proteins incubated with (+CK2) or without (-) human recombinant CK2. 1pmol fractions of protein samples from **(B)** that were produced in *E. coli*, HisTrap purified and incubated with or without 250 U of CK2 for 3 hours at 30ºC, were additionally incubated with the fluorophore-coupled *LS7* DNA probe in EMSA buffer for 20 minutes in the dark. These were subsequently electrophoresed for 80 minutes at 130 V in a 4 % EMSA gel. The fluorescent DNA probe was detected by the Odyssey imager. The DNA probe alone was detected at the bottom of the gel, while the DNA-protein complex displayed decreased mobility. Δ12TGA2 and Δ24TGA2 stopped binding to DNA after CK2 treatment, whereas Δ43TGA2 did not.

(D) Immunoblot analyses of *E. coli* expressed and HisTrap purified Δ12TGA2, Δ24TGA2 and Δ43TGA2 proteins treated with or without CK2 and analysed by size exclusion chromatography. Fractions corresponding to the oligomeric state found in the void volume (Lane 1) along with those eluted within the included volume corresponding to protein tetramers, trimers, dimers and monomers (Lanes 2-5, respectively) were acetone precipitated, electrophoresed and transferred to a nitrocellulose membrane. Proteins of interest were detected via a primary anti-His-tag antibody and a secondary fluorophore-coupled antibody using the Odyssey imager. All 3 analysed proteins formed oligomers but could also form smaller complexes after CK2 treatment.

4.4 Deletions of the TGA2 N-terminus affect the phosphorylation rate

A highly interesting observation was made when performing EMSA experiments using the N-terminal deletion mutants of TGA2. In a nearly identical experimental scenario as in Figure 3C, ten-fold less of enzyme (25 U) was used to determine the efficiency of protein phosphorylation. As seen in Figure 4, full length TGA2 was substantially phosphorylated resulting in a considerable decrease of band intensity of the retarded protein-DNA complex, while the intensity decrease of the others was not as pronounced. Using LI-COR Image Studio software (Version 2.1.10), the complex intensities in samples treated or untreated with CK2 were obtained. Their comparisons revealed that 95% of TGA2 stopped binding to DNA due to phosphorylation, while the same effect was seen only in 52, 24 and 1% of Δ12TGA2, Δ24TGA2 and Δ43TGA2, respectively. Due to the fact that no decrease was observed with Δ43TGA2 in prior experiments, the minute 1% decrease is probably not caused by phosphorylation

but can be most likely attributed to technical error. More importantly, this result illustrates the significance of the N-terminal sequence for facilitating interaction of TGA2 with the enzyme. Without it, the reaction occurs at a significantly slower pace.

This observation could also explain the contradictions our data pose to findings previously published by Kang and Klessig (2005). Using radioactively labelled ATP substrate and TGA2 deletion mutants lacking the first 20 or 40 amino acids, Kang and Klessig (2005) detected minimal incorporation of radioactive phosphate into these mutants compared to wild type TGA2 and, therefore, concluded that the CK2 target sites are located only among the first 20 amino acids. Our results clearly contradict this statement but also provide an explanation: the fact that the efficientcy of phosphorylation of TGA2 N-terminal deletion mutants is significantly decreased (to only 24% in our Δ24TGA2), along with the decrease of radioactive signal due to the presence of less target residues, would indeed account for very low detection of TGA2 N-terminal deletion mutants exposed to CK2 when compared to the full length version.

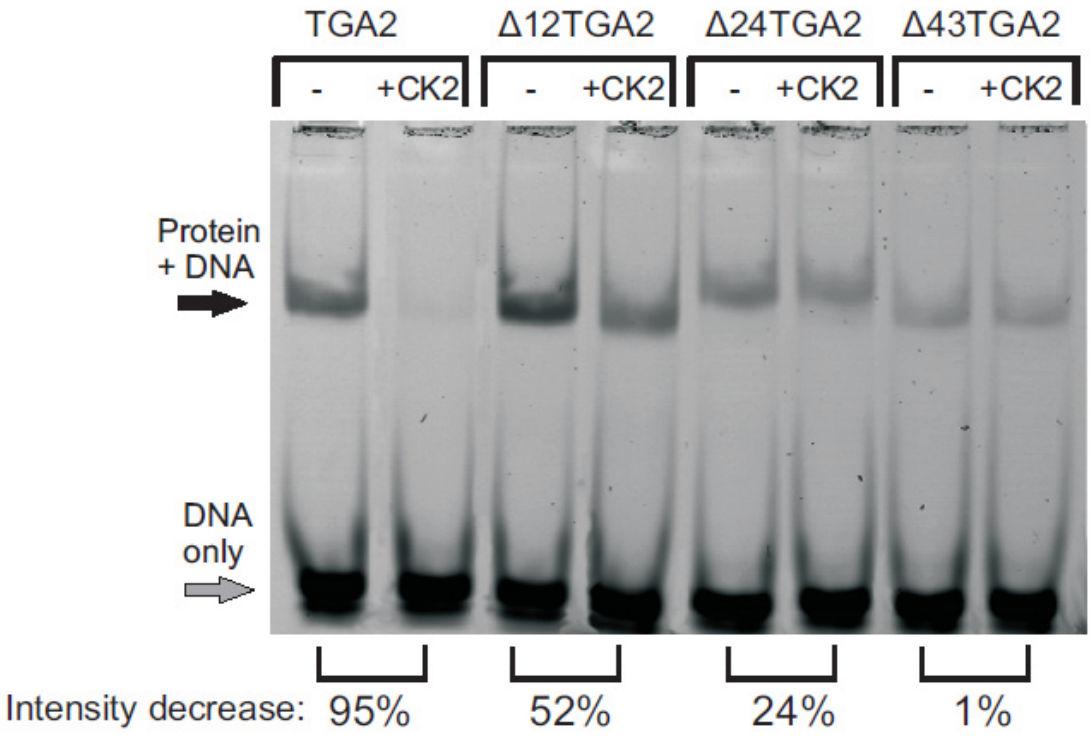

Figure 4. Phosphorylation by CK2 is less effective when using TGA2 N-terminal deletion mutants.

Electromobility shift assay using full length TGA2, Δ12TGA2, Δ24TGA2 and Δ43TGA2 proteins incubated with (+CK2) or without (-) human recombinant CK2. These proteins were produced in *E. coli*, HisTrap purified and incubated with or without 25 U of CK2 for 3 hours at 30ºC. Additionally, they were incubated with the fluorescent *LS7* DNA probe in EMSA buffer for 20 minutes in the dark. These samples were subsequently electrophoresed for 80 minutes at 130 V in a 4 % EMSA gel. The fluorescent DNA probe was detected by the Odyssey imager. The DNA probe alone was detected at the bottom of the gel, while the DNA-protein complex displayed decreased mobility. Intensities of the protein-DNA complexes were measured using the LI-COR Image Studio software (Version 2.1.10) and those of samples untreated or treated with CK2 were compared. The relative intensity decreases of these complexes are included.

4.5 Mass analysis of phosphorylated TGA2 reveals 13 target residues

Due to the fact that we were unable to narrow down the search for phosphorylation sites using substitution or deletion TGA2 mutants, we chose to identify residues modified by CK2 via mass spectrometry. Briefly, 3 samples each containing 5 nmol of affinity purified TGA2 were incubated with recombinant human CK2. All protein samples were then acetone precipitated, separated using SDS-PAGE and visualized using Coomassie Brilliant Blue. Three bands corresponding to phosphorylated TGA2 were excised from the gel, frozen and analysed at Harvard Mass Spectrometry and Proteomics Resource Laboratory, FAS Center for Systems Biology, Cambridge MA. Each of the three samples was subjected to protease digestion and then analysed by microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry (μLC/MS/MS) conducted on a Thermo LTQ-Orbitrap mass spectrometer. The addition of a single phosphate to a target residue results in increased molecular mass of a peptide by 79.9663g/mol. Mass analysis of peptides post digestion is capable of pinpointing the phosphorylated residues of a protein.

Surprisingly, a total of 13 phosphorylated TGA2 residues were identified. Figure 5 is a schematic depicting all phosphorylated residues within TGA2 (for a more detailed report, see Supplemental Figure 2). The majority of CK2 targets are indeed in the N-terminus and these include the previously identified motif containing Ser^{11}, Thr^{12} and Thr^{16}. The presence of 10 phosphorylated N-terminal residues demonstrates the importance of this domain as the primary target of

protein kinase CK2. Interestingly, not all serines and threonines in this domain are modified, as can be seen with Thr^{4} and Ser^{5}.

Our previous analyses of N-terminal deletion mutants suggested that the residues which negatively regulate the ability of the bZIP domain of the TGA2 transcription factor to bind DNA are most likely located between the 25^{th} and 43^{rd} amino acid. Mass analysis revealed that there are five phosphorylated residues in this region (Thr^{30}, Ser^{33}, Ser^{35}, Ser^{36} and Ser^{39}). These were not identified by Kang and Klessig (2005) and we investigated their biological significance in subsequent experiments.

Additionally, three more targeted residues were found downstream of the N-terminal domain. One is located within the leucine zipper motif (Thr^{80}), while the remaining two are in the C-terminal domain (Ser^{143} and Ser^{315}). These residues must be responsible for oligomer disassembly, since they are the only modified amino acids left in the Δ43TGA2 mutant, which is capable of forming smaller complexes and monomers after incubation with CK2 (Figure 3D).

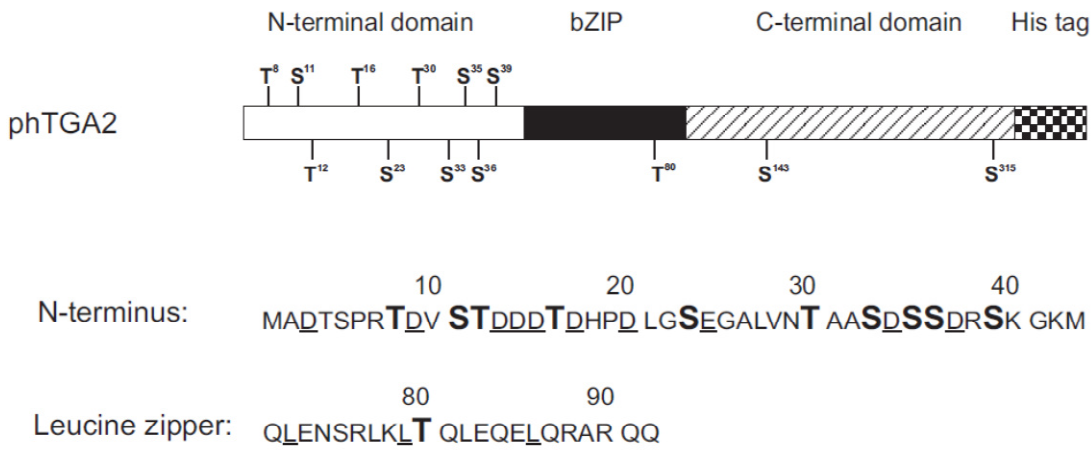

Figure 5. Phosphorylated TGA2 residues identified by mass analysis.

Schematic representation of the His-tagged full length TGA2 treated with human recombinant CK2 (phTGA2) depicting 13 phosphorylated residues found within all TGA2 domains (the N-terminal domain, basic domain/leucine zipper bZIP and C-terminal domain). Also included are the amino acid sequences of the N-terminus and leucine zipper dimerization motif with phosphorylated residues in bold and larger font size. Underlined within the N-terminal sequence are the aspartate (D) and glutamate (E) residues often found in CK2 target sequences, while the 3 leucines (L) underlined in the leucine zipper sequence are those important for forming this dimerization motif.

4.6 TGA2 mutants lacking portions of the leucine zipper domain lose their DNA binding ability after CK2 treatment but do not deoligomerize

The knowledge of CK2 target residues within TGA2 enabled us to hypothesize which of these are actually responsible for the loss of DNA binding abilities or protein deoligomerization. Interestingly, one targeted threonine (Thr^{80}) is located in the middle of the leucine zipper motif directly beside the middle

leucine involved in forming this dimerization motif (Figure 5). The addition of two phosphate groups (one for each TGA2 molecule involved) could have a profound impact on the dimerization function due to repulsion via their negative charges.

To determine the significance of this particular amino acid, we used a set of His-tagged TGA2 mutants that lacked portions of the leucine zipper motif (Figure 6A). These lacked 19, 13 and 6 amino acids found upstream of the 93rd TGA2 amino acid (TGA2Δ73-92, TGA2Δ80-92 and TGA2Δ87-92, respectively). First, we tested their DNA-binding capabilities using EMSA. A noticeable trend appeared: the longer the deleted sequence, the lower DNA binding compared to full length TGA2 (Figure 6B). In this experiment, no DNA-protein complex was detected when using TGA2Δ73-92 (Figure 6B, lane 2). However, the leucine zipper is not directly responsible for DNA binding of the bZIP domain; that is the function of the preceding basic sequence. Nevertheless, the presence of the bZIP domain in a large number of transcription factors suggests that both motifs are important for proper function. Use of higher amounts of TGA2Δ73-92, along with increased detection intensity, revealed that this protein can also bind DNA but at a very low level (Figure 6C, lane 5). Therefore, it seems that the dimerizing leucine zipper is indeed important, but not crucial, for DNA binding. However, it may be important for proper spatial orientation of the basic sequence increasing its affinity to DNA. Furthermore, all three proteins were treated with human recombinant CK2 with the same result: loss of DNA binding. Immunoblot analysis

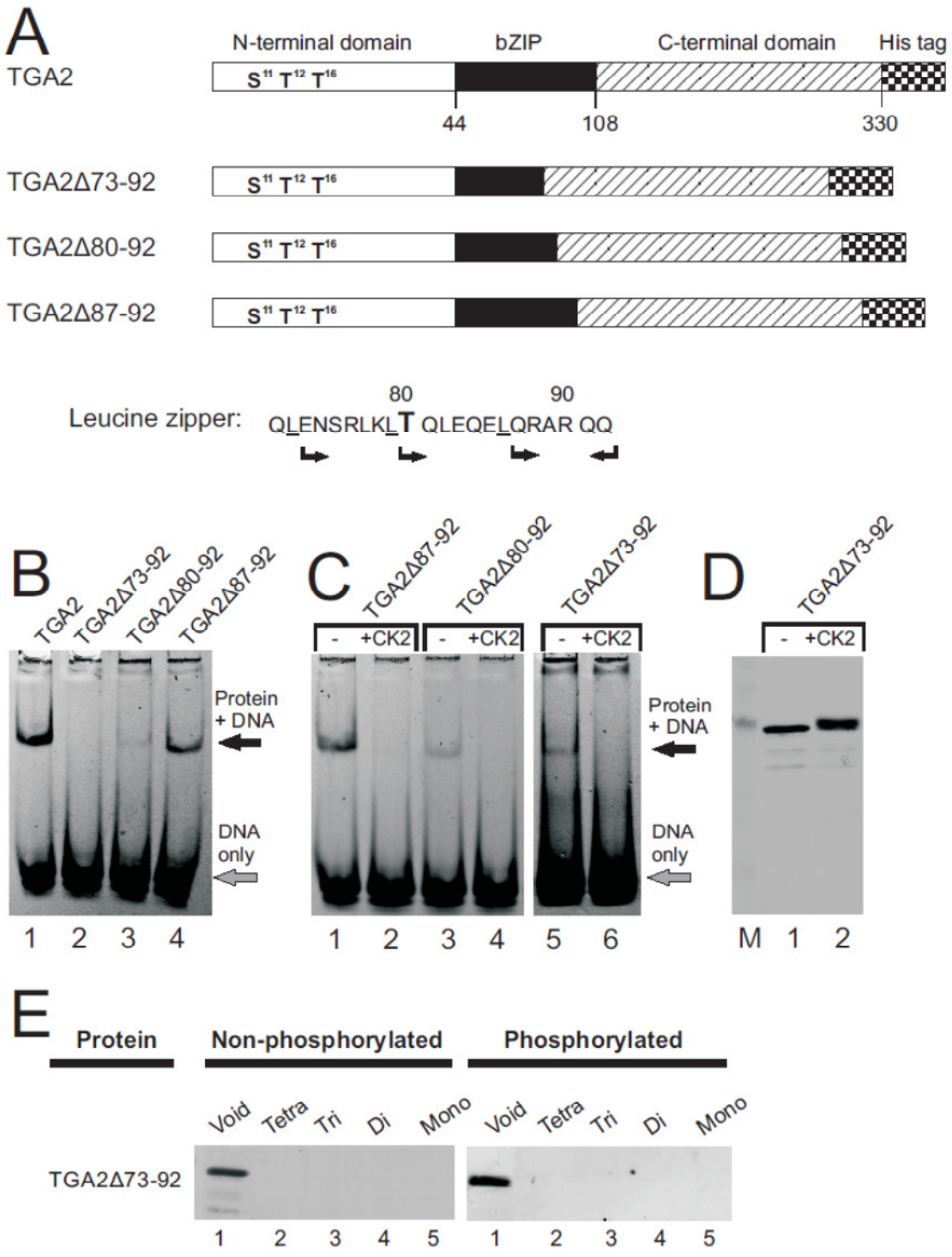

Figure 6. Leucine zipper-mutated TGA2 proteins are incapable of binding DNA and form only oligomers after CK2 phosphorylation.

(A) Schematic representation of proteins tested for CK2 phosphorylation in **(B-E)**, namely the His-tagged full length TGA2 and the leucine zipper deletion mutants lacking 19, 13 and 6 amino acids from this motif (TGA2Δ73-92, TGA2Δ80-92 and TGA2Δ87-92, respectively). TGA2's N-terminal domain, basic domain/leucine zipper (bZIP) and C-terminal domain, as well as the His tag, are also depicted along with the numbers corresponding to starting amino acids of these domains. Additionally, the sequence of the TGA2 leucine zipper motif is included with arrows indicating the beginning and end of the deleted sequences. Leucines involved in the formation of this motif are underlined, whereas the phosphorylated threonine (T^{80}) is in bold and larger font size.

(B) Electromobility shift assay using His-tagged TGA2, TGA2Δ73-92, TGA2Δ80-92 and TGA2Δ87-92. These proteins were produced in *E. coli*, HisTrap purified and identical amounts incubated with the fluorescent *LS7* DNA probe in EMSA buffer for 20 minutes in the dark. These samples were subsequently electrophoresed for 80 minutes at 130 V in a 4 % EMSA gel. The fluorescent DNA probe was detected by the Odyssey imager. The DNA probe alone was detected at the bottom of the gel (grey arrow), while the DNA-protein complex displayed decreased mobility (black arrow). A decrease in DNA binding was observed when proteins lacking longer leucine zipper sequences were tested.

(C) Electromobility shift assay using His-tagged TGA2, TGA2Δ73-92, TGA2Δ80-92 and TGA2Δ87-92 incubated with (+CK2) or without (-) human recombinant CK2. These proteins were produced in *E. coli*, HisTrap purified and incubated with or without 250 U of CK2 for 3 hours at 30ºC. Fractions of these samples were subsequently incubated with the *LS7* DNA probe in EMSA buffer for 20 minutes in the dark, and electrophoresed for 80 minutes at 130 V in a 4 % EMSA gel. The fluorescent DNA probe was detected by the Odyssey imager. The DNA probe alone was detected at the bottom of the gel (grey arrow), while the DNA-protein complex displayed decreased mobility (black arrow). All tested proteins were affected by phosphorylation, including TGA2Δ73-92 where higher protein

amounts and higher detection intensity had to be used to visualize the DNA-protein complex.

(D) Immunoblot analysis of the TGA2Δ73-92 protein incubated with (+CK2) or without (-) human recombinant CK2. This protein was produced in *E. coli*, HisTrap purified and incubated with or without 250 U of CK2 for 3 hours at 30ºC. The protein samples were acetone precipitated, separated using 12% SDS-PAGE and transferred to a nitrocellulose membrane. Proteins of interest were detected via a primary anti-His-tag antibody and a secondary fluorophore-coupled antibody using the Odyssey imager. A difference in migration of unphosphorylated and phosphorylated protein (Lanes 1 and 2, respectively) can be observed. Lane M contains the protein standard marker.

(E) Immunoblot analyses of *E. coli* expressed and HisTrap purified TGA2Δ73-92 protein incubated without or with CK2 and analysed by size exclusion chromatography. Fractions corresponding to the oligomeric state found in the void volume (Lane 1) along with those in the included volume corresponding to protein tetramers, trimers, dimers and monomers (Lanes 2-5, respectively) were acetone precipitated, electrophoresed and transferred to a nitrocellulose membrane. Proteins of interest were detected via a primary anti-His-tag antibody and a secondary fluorophore-coupled antibody using the Odyssey imager. Both experiments yielded oligomers but no smaller complexes were detected.

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confirmed via protein mobility shift that TGA2Δ73-92 is indeed phosphorylated (Figure 6D) and that the loss of DNA binding seen in EMSA is caused by this protein modification. Hence, Thr^{80} is not the residue responsible for abolishing TGA2's interaction with DNA.

Additionally, phosphorylation of the TGA2Δ73-92 protein lacking the entire leucine zipper sequence was also studied using gel filtration analysis.

Nonphosphorylated TGA2Δ73-92 formed only oligomers (Figure 6E, Void fraction), the same as all other nonphosphorylated deletion mutant proteins studied thus far (compare with Figure 3D). However, incubation with CK2 did not result in deoligomerization as in the previous cases as only oligomers were detected (Figure 6E). This result implies that phosphorylation of Thr^{80} may be responsible for protein deoligomerization.

4.7 Mutations of phosphorylatable residues found between positions 30 and 39 disable DNA binding regulation by CK2

Alterations of TGA2's affinity to DNA due to phosphorylation have been known for some time (Hildago et al., 2001; Kang and Klessig, 2005). However, the mechanism behind this change has not been addressed until now. The most plausible explanation would have been that the bZIP motif itself is targeted by CK2, which would abolish the interaction with DNA. Nonetheless, no phosphorylated residues were detected by mass analysis in the basic region of this motif and the possibility that DNA regulation is mediated by the phosphorylated threonine inside the leucine zipper was disproven by our previous experiment (Figure 6C).

Therefore, we turned our focus back to the TGA2 N-terminus which neighbours the basic region and harbours many CK2 target residues. The fact that the N-terminal deletion of 43 amino acids disrupts regulation of DNA binding, while deletion of only 24 residues does not, hinted at the position of the regulatory motif. Mass analysis revealed that five residues are targeted by CK2 here (Thr^{30}, Ser^{33}, Ser^{35}, Ser^{36} and Ser^{39}, Figure 5). For this reason, we

substituted these amino acids by alanines in the His-tagged TGA2 and named the resulting protein TGA2-5A (Figure 7A). We tested DNA binding of this protein treated with or without the kinase and saw no significant difference between the two samples (Figure 7B). Western blot analysis revealed that phosphorylated TGA2-5A migrates slower than its nonphosphorylated counterpart in SDS-PAGE (black arrow, Figure 7C) proving that TGA2-5A is indeed modified by CK2 but this modification doesn't have an effect on its DNA binding. Of note, the multiple alanine substitutions have a large impact on this protein's resilience to *E. coli* proteases. The majority of TGA2-5A is cleaved in bacterial cells into smaller fragments one of which is co-purified and detected by the anti-His-tag antibody and displays a molecular weight of around 25 kDa (grey arrow, Figure 7C). Based on this estimate of molecular weight, we can assume that the cleavage site is most likely at the end of the leucine zipper portion of the bZIP domain or at the beginning of the C-terminal domain. Fortunately, this cleaved product originating from TGA2-5A's C-terminus would be unable to interact with DNA as it does not contain the basic domain required for DNA binding and probably also lacks a good portion of the leucine zipper. Therefore, this C-terminal product should not have any impact on the EMSA assay. The N-terminal cleavage product could pose a problem to the assay but it would not be co-purified by a HisTrap column as it doesn't contain the His tag and thus would not found in the purified protein sample. In the end, the yield of the full length protein is very low and highly contaminated by the C-terminal cleaved product. Regardless of this complication,

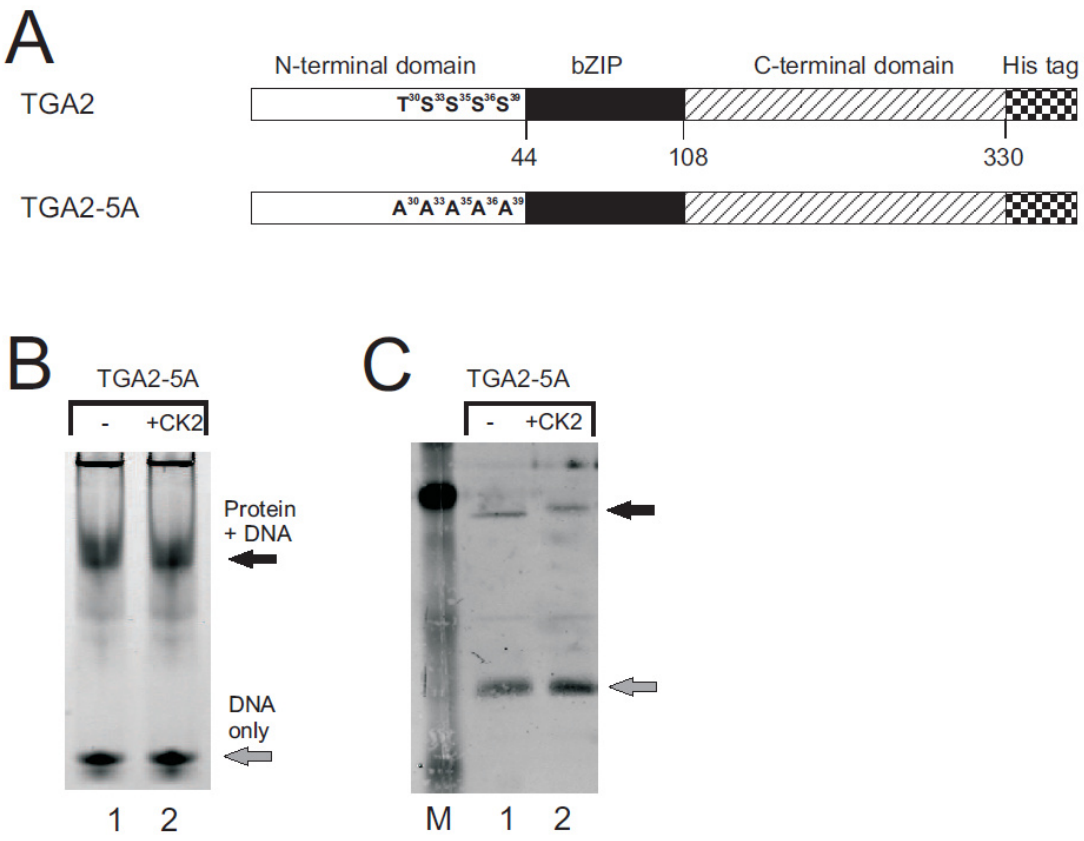

Figure 7. Phosphorylation of residues found between positions 30 and 39 regulates TGA2's DNA binding ability.

(A) Schematic representation of the His-tagged full length TGA2 and its mutant with Thr^{30}, Ser^{33}, Ser^{35}, Ser^{36} and Ser^{39} replaced by alanines which was tested for CK2 phosphorylation in **(B)** and **(C)**. TGA2's N-terminal domain, basic domain/leucine zipper (bZIP) and C-terminal domain, as well as the His tag, are also depicted along with the numbers corresponding to starting amino acids of these domains.

(B) Electromobility shift assay using His-tagged TGA2-5A incubated with (+CK2) or without (-) human recombinant CK2. This protein was produced in *E. coli*, HisTrap purified and incubated with or without 250 U of CK2 for 1 hour at 30ºC. Fractions of these samples were subsequently incubated with the *LS7* DNA probe in EMSA buffer for 20 minutes in the dark, and electrophoresed for 80

minutes at 130 V in a 4 % EMSA gel. The fluorescent DNA probe was detected by the Odyssey imager. The DNA probe alone was detected at the bottom of the gel (grey arrow), while the DNA-protein complex displayed decreased mobility (black arrow). No significant difference of TGA2-5A's affinity to DNA was observed.

(C) Immunoblot analysis of the TGA2-5A protein incubated with (+CK2) or without (-) human recombinant CK2. This protein was produced in *E. coli*, HisTrap purified and incubated with or without 250 U of CK2 for 1 hour at 30ºC. The protein samples were acetone precipitated, separated using 12% SDS-PAGE and transferred to a nitrocellulose membrane. Proteins of interest were detected via a primary anti-His-tag antibody and a secondary fluorophore-coupled antibody using the Odyssey imager. A difference in migration of unphosphorylated and phosphorylated protein (black arrow, lanes 1 and 2, respectively) can be observed. Grey arrow points to a TGA2-5A fragment generated by bacterial proteases. Lane M contains the protein standard marker.

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the TGA-5A protein's DNA affinity seems to be unaffected by the kinase meaning that this motif is responsible for regulating how the basic domain of TGA2 interacts with DNA. This conclusion is supported by the proximity of the two structures. The regulatory motif is found between the 30th and 39th amino acid and the basic region starts with the 44th residue of TGA2. Therefore, structural changes of one of these could easily influence the other resulting in abolishing DNA binding of TGA2.

Due to the aforementioned complications, gel filtration analysis was not performed as the amount of full length protein would be insufficient for detection

and would further decrease as the protein is continuously processed by proteases contaminating the sample.

4.8 The POZ domain of NPR1 restores the DNA binding ability of phosphorylated TGA2

The N-terminus of TGA2 acts as a repression domain *in vivo* which causes TGA2 to function as a *PR1* repressor prior to pathogen infection or SA treatment (Boyle et al., 2009). However, the POZ domain of NPR1 is capable of negating this function by directly interacting with TGA2's N-terminus. This is also a region of TGA2 that is heavily phosphorylated by CK2 (Figure 5), therefore, we asked if the interaction between TGA2 and POZ is altered when TGA2 is phosphorylated.

To answer this question, we performed an EMSA experiment using E. coli produced and His-trap purified TGA2 and POZ proteins. 100 pmols of TGA2 were incubated with 250U of CK2 for 30 minutes at 30ºC. A 2 pmol fraction of phosphorylated TGA2 was incubated with 5 pmols of POZ and the fluorescent *LS7* DNA probe in EMSA buffer for 20 minutes in the dark. These samples were then separated by gel electrophoresis and the migration of the probe was detected by fluorescence. POZ had a positive effect on nonphosphorylated TGA2 as it enhanced DNA binding (Figure 8, lane 2). More surprisingly, the addition of POZ restored the DNA binding ability of CK2 treated TGA2 (Figure 8, lane 4). Therefore, POZ is capable of negating the effect of CK2 on the DNA binding

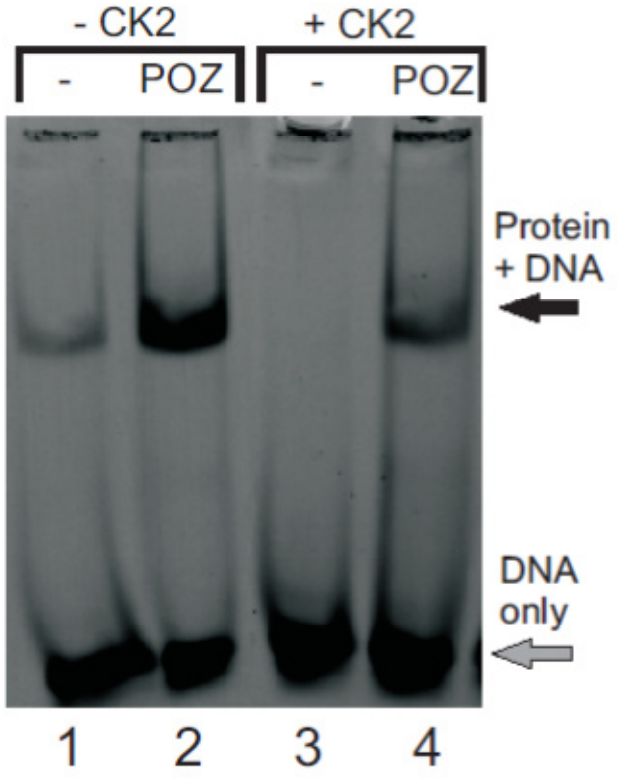

Figure 8. NPR1 POZ restores DNA binding of phosphorylated TGA2.

Electromobility shift assay using His-tagged TGA2 incubated with (+CK2) or without human recombinant CK2, and subsequently incubated without (-) or with the His-tagged POZ domain of NPR1 (POZ). His-tagged proteins were produced in *E. coli* and HisTrap purified. 100pmol of TGA2 was incubated with or without 250 U of CK2 for 30 minutes at 30ºC. Fractions of these samples were subsequently incubated with the NPR1 POZ protein and the *LS7* DNA probe in EMSA buffer for 20 minutes in the dark, and electrophoresed for 80 minutes at 130 V in a 4 % EMSA gel. The fluorescent DNA probe was detected by the Odyssey imager. The DNA probe alone was detected at the bottom of the gel (grey arrow), while the DNA-protein complex displayed decreased mobility (black arrow). Addition of the POZ protein increased the DNA binding of unphosphorylated TGA2 (Lane 2) and restored the DNA binding of phosphorylated TGA2 (Lane 4).

ability of TGA2. This information further supports the idea that it is not the leucine zipper or C-terminal phosphorylatable residues that are responsible for regulating DNA binding, but rather those found in the N-terminus since the POZ domain of NPR1 interacts with this TGA2 region (Boyle et al., 2009).

4.9 Recombinant CK2α from Arabidopsis interacts with and phosphorylates TGA2 and is capable of autophosphorylation

CK2α isoforms in various organisms are quite similar, the kinase core responsible for catalytic activity and substrate recognition being highly conserved among species (Guerra and Issinger, 1999). We wanted to test if the Arabidopsis CK2 has the same effect on TGA2 as the human isoform. To do so, we generated CK2α1 constructs containing an affinity tag. Recombinant CK2α proteins have been shown to be catalytically active *in vitro* without the presence of regulatory β subunits (Allende and Allende, 1995), therefore, these constructs alone should be capable of phosphorylating TGA2. We produced a CK2α1 protein fused to an N-terminal Strep-tag in *E. coli*, affinity purified it using a Strep-Tactin Superflow Plus column, and utilized the eluted protein for TGA2 phosphorylation. Subsequent EMSA analysis clearly shows that the Arabidopsis isoform possesses enzymatic activity, as TGA2 proteins display decreased binding to DNA after incubation with the kinase (Figure 9A). Purified CK2α1 is capable of making use of both ATP and GTP phosphate donors, although the reaction occurs much more rapidly with the former (compare lanes 3 and 4 in Figure 9A). This result is in accordance with previous findings, since CK2 is one of a few kinases that accept GTP as a substrate (Tuazon and Traugh, 1991).

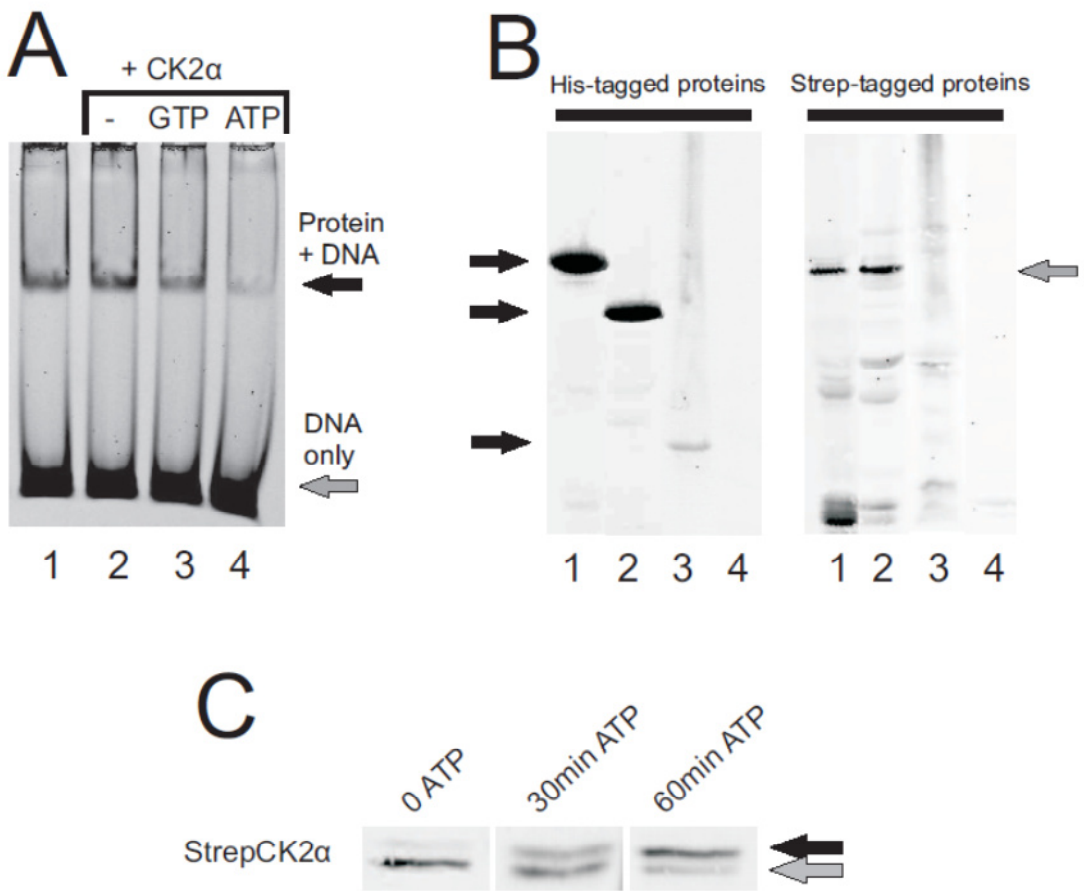

Figure 9. Arabidopsis recombinant CK2α interacts with and thus phosphorylates TGA2 and is capable of autophosphorylation.

(A) Electromobility shift assay using His-tagged TGA2 and Arabidopsis recombinant CK2α. These proteins were produced in *E. coli* and HisTrap purified. TGA2 was incubated without or with CK2α along with ATP, GTP or without any nucleotides for 30 minutes at room temperature. Samples were then incubated with the fluorescent *LS7* DNA probe in EMSA buffer for 20 minutes in the dark. These samples were subsequently electrophoresed for 80 minutes at 130 V in a 4 % EMSA gel. The fluorescent DNA probe was detected by the Odyssey imager. The DNA probe alone was detected at the bottom of the gel (grey arrow), while the DNA-protein complex displayed decreased mobility (black arrow). A significant decrease in DNA binding was observed when CK2α and ATP were added.

(B) Pull-down experiments testing the interaction of column-bound His-tagged proteins with Strep-tagged Arabidopsis recombinant CK2α. All proteins were produced in *E. coli*. Bacterial lysates containing the His-tagged TGA2, Δ43TGA2 and NPR1 POZ were retained by a HisTrap column. Subsequent loading of the CK2α-containing bacterial lysate allowed for CK2α binding to interacting proteins on the column. Non-interacting proteins were washed out and the retained proteins were subsequently eluted. These fractions were then acetone precipitated, electrophoresed and transferred to a nitrocellulose membrane. Proteins of interest were detected on the same immunoblot either via a primary anti-His-tag mouse antibody (Left image, black arrows) or an anti-Strep-tag rabbit antibody (Right image, grey arrow) and secondary fluorophore-coupled antibodies using the Odyssey imager. CK2α interacted only with TGA2 (Lane 1) and Δ43TGA2 (Lane 2), no kinase was detected when NPR1 POZ (Lane 3) was used or when no His-tagged protein (Lane 4) was used.

(C) Immunoblot analysis of the Strep-tagged CK2α protein incubated on-column with ATP for various time periods. This protein was produced in *E. coli*, Streptactin purified and incubated on-column without or with ATP for 30 and 60 minutes. Eluted protein samples were acetone precipitated, separated using 12% SDS-PAGE and transferred to a nitrocellulose membrane. Proteins of interest were detected via a primary anti-Strep-tag antibody and a secondary fluorophore-coupled antibody using the Odyssey imager. Two detected bands correspond to the autophosphorylated (black arrow) and non-phosphorylated (grey arrow) forms of CK2α. With increasing incubation time, the amount of autophosphorylated form increased, while the non-phosphorylated form decreased.

Since CK2α1 phosphorylates full length TGA2, we were interested if it also interacts with Δ43TGA2, despite the fact that this deletion mutant is devoid of the N-terminus and thus lacks the acidic regions necessary for substrate recognition

by CK2. Since there is no substantial effect of human recombinant CK2 on DNA binding abilities of Δ43TGA2, we could not use EMSA to detect such an interaction. Therefore, we switched over to on-column pull-down assays. The idea behind this method is that a Strep-tagged protein will be retained by the HisTrap^{TM} HP column only if it interacts with a His-tagged protein already bound to the column's resin. Simply, the steps in this technique were to bind a His-tagged protein to the HisTrap^{TM} HP column, wash away any unbound proteins, load a cellular lysate containing the Strep-tagged protein, wash again and finally elute all proteins. The eluted fractions were then acetone precipitated, separated by SDS-PAGE and immunobloted. Detection of the two proteins of interest is enabled by anti-His-tag or anti-Strep-tag antibodies. In all our experiments, the Strep-tagged protein was always StrepCK2α1. This method enabled us to conclude that Arabidopsis CK2α1 is capable of interacting with Δ43TGA2 (Figure 9B), which was phosphorylated by the human isoform (Figures 3B and 3D). Therefore, it seems that the N-terminus is not required for the interaction of TGA2 with the kinase.

Concerns dealing with the possibility of non-specific interaction of the Strep-tagged CK2α1 with the column resin were addressed in subsequent pull-down experiments, in which no His-tagged protein was used (Figure 9B). In this scenario, no CK2α1 was detected and, therefore, this experiment served as a negative control. Also, no CK2 α1 was detected when we used the POZ domain of NPR1 in the pull-down experiment illustrating that the previously detected interaction of TGA2 with the kinase is not caused by nonspecific protein

interactions. Additionally, this shows that the POZ domain is not capable of interacting with CK2 and, therefore, not able to recruit the kinase to TGA2 showing that CK2 functions independently of POZ.

In vitro purified CK2α proteins can exhibit autophosphorylation, an event investigated in a study by Donella-Deana et al. (2001) which discovered that human CK2α subunits undergo intermolecular tyrosine autophosphorylation. During our Strep-Tactin Superflow Plus purification procedures, column-bound CK2α1 was washed and incubated with buffer containing ATP prior to elution. As seen in Figure 9C, two forms of CK2α1 represented by two bands were detected by consequent immunoblot analysis. Without the presence of ATP, the majority of protein is found in the bottom band (grey arrow). On the other hand, incubation with ATP for 30 and 60 minutes results in an intensity increase of the slower moving top band (black arrow) compensated by an intensity decrease of the lower band. In our previous immunoblot analyses, slight mobility shifts of proteins in SDS-PAGE were attributed to protein phosphorylation, an event also seen, for example, in the case of TGA2 (Figure 1B). Therefore, the change of CK2α1 migration is most likely due to similar autophosphorylation events as described by Donella-Deana et al. (2001). In conclusion, Arabidopsis CK2α1 seems to have very similar characteristics as its human counterpart.

4.10 CK2α is localized to the *PR1* promoter *in vivo*

Our data clearly show that both human recombinant CK2 and Arabidopsis recombinant CK2α1 phosphorylate TGA2 thus changing its affinity to DNA *in vitro*. We also tested if this event happens *in vivo*. Arabidopsis leaf extracts possess the CK2-like activity needed to phosphorylate TGA2 (Kang and Klessig, 2005) but this information is not sufficient to prove that CK2 is the responsible kinase as there are more than 1000 protein kinase genes found in the genome of *Arabidopsis thaliana* (Nemoto et al., 2011). TGA2 is constitutively localized to the *PR1* promoter (Rochon et al., 2006); therefore, we investigated if CK2 is localized to this region as well.

Chromatin immunoprecipitation (ChIP) is a technique widely used to investigate DNA-protein interactions *in vivo*. Briefly, plant tissue is first soaked in a formaldehyde solution. This chemical penetrates cells and chemically cross-links basic residues of proteins to near bases of DNA. Therefore, proteins directly or indirectly interacting with DNA will be covalently interconnected with the nucleic acid (Massie and Mills, 2008). In the next step, plant cells are disrupted and the soluble protein fraction is sonicated. Ultra-sound will shear DNA that is not protected by fixed proteins yielding smaller DNA-protein complexes. The sonicated sample is then incubated with Protein A beads displaying an antibody of researcher's choice on their surface. In this experiment, it was a polyclonal antibody raised against an epitope corresponding to amino acids 2-320 of the human CK2α. As mentioned previously, the catalytic core of CK2α isoforms is conserved (see Supplemental Figure 3) and, therefore, this

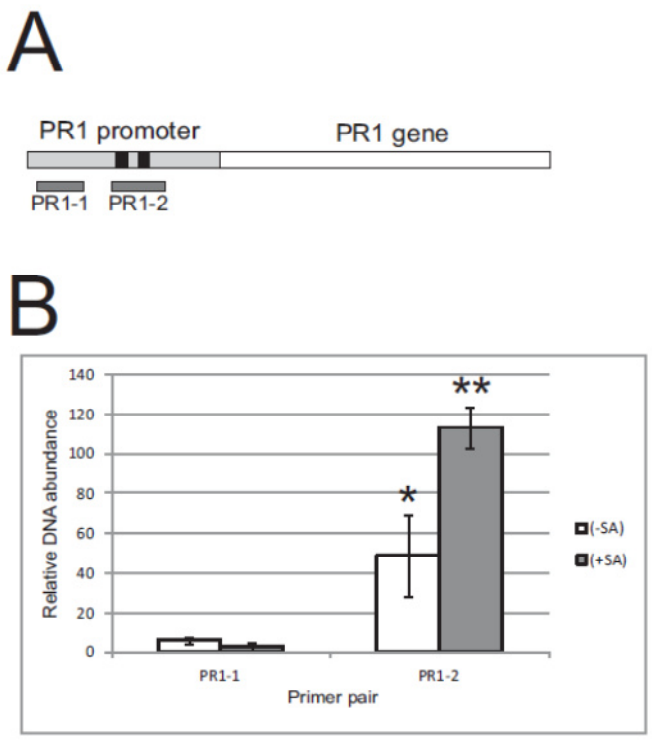

Figure 10. CK2α localization on the *PR1* promoter.

(A) Schematic depiction of the *PR1* promoter (light grey), *PR1* gene (white) and PCR products obtained when using primer pairs PR1-1 and PR1-2 (dark grey). Two TGA2 binding sites within the *PR1* promoter are depicted in black.

(B) Histogram illustrating CK2α localization on the *PR1* promoter. Formaldehyde fixed leaf tissue treated with or without SA from 6 week old Arabidopsis plants was used for ChIP experiments using an anti-CK2α polyclonal antibody to co-precipitate DNA in close vicinity of this protein. This DNA was purified and used in qPCR reactions with the PR1-1 and PR1-2 primer pairs. Values obtained were standardized to the abundance of the co-precipitated *ubiquitin 10* gene. The final values are averages of 3 biological replicates; the error bars display their standard deviation. The significantly higher DNA abundance was detected using the PR1-2 primer pair compared to PR1-1 (denoted by asterisk, $P < 0.05$), and there was a significant difference between the PR1-2 obtained DNA from SA-treated and non-treated tissue (double asterisk, $P < 0.05$).

antibody is able to detect the Arabidopsis CK2α (see Supplemental Figure 4). The antibody-covered beads bind the target protein (here the CK2α isoforms) along with the attached DNA fragments, which are released only after incubation with SDS. These isolated fragments are subsequently precipitated and the amount of DNA is determined by quantitative PCR (qPCR).

For determining if CK2α actually interacts with the *PR1* promoter, PCR primers complementary to the *PR1* sequence must be used. In this experiment, we used two sets of primer pairs: one pair targets a sequence embedded in the middle of the promoter containing the region to which TGA transcription factors bind (primer pair PR1-2, see Figure 10A), while the second pair targets a promoter region further away from the TGA-binding sites (primer pair PR1-1). Values obtained via qPCR were standardized to the abundance of the co-precipitated *ubiquitin 5* gene. As seen in Figure 10B, the amount of DNA isolated via ChIP and detected using the PR1-2 primer pair was significantly increased compared to the amount detected by PR1-1. Therefore, there is much more CK2α interacting with the PR1 promoter in the area where TGA2 binding sites are located. Additionally, significantly more DNA was detected by the PR1-2 primer pair when the plant tissue was treated by SA. This would suggest that the kinase is enriched at the PR1 promoter during SAR.

In conclusion, CK2α can interact with the *PR1* promoter proving that this kinase is localized to the same region as the TGA2 transcription factor *in vivo* thus supporting its probable role in TGA2 regulation and SAR.

CHAPTER 5 - DISCUSSION - BIOLOGICAL SIGNIFICANCE OF TGA2 PHOSPHORYLATION

Although TGA2 phosphorylation by protein kinase CK2 had been investigated in an earlier study (Kang and Klessig, 2005), this work provides an in-depth analysis of this event *in vitro* and investigates its molecular consequences within systemic acquired resistance (SAR). Firstly, we show that the previously identified phosphorylated residues found in TGA2's N-terminus (Ser^{11}, Thr^{12} and Thr^{16}) are not the only ones targeted by CK2 as mutants lacking these particular phosphorylation sites are still affected by the kinase (Figure 1). Secondly, we managed to identify all residues phosphorylated by human recombinant CK2 via mass spectrometry, 10 of which are within the N-terminus, one is in the leucine zipper motif of the bZIP domain and two are in the C-terminal domain (Figure 5). Thirdly, we observed that phosphorylation of TGA2 leads to two events altering the protein's interaction with other molecules: a decrease in affinity to DNA containing the *LS7* binding sequence (Figure 1) and a partial deoligomerization of TGA2 complexes (Figure 2). Fourthly, these two events seem to be mediated by two separate protein regions since a TGA2 mutant lacking the first 43 N-terminal amino acids deoligomerizes but its binding to DNA is unchanged after CK2 treatment (Figure 3), whereas kinase treatment of a TGA2 mutant lacking the leucine zipper motif abolishes its DNA binding but does not cause protein deoligomerization (Figure 6). Thus, we determined that DNA affinity is modulated by modification within a multiserine motif found in the N-terminus, while deoligomerization is most likely caused by phosphorylation of a

threonine in the leucine zipper. Fifthly, we observed the same effect on TGA2 when an Arabidopsis recombinant CK2α enzyme was used (Figure 8) suggesting that the two CK2α isoforms originating from various organisms seem to function in a similar manner possibly due to their very high sequence similarity. Finally, *in vivo* experiments showed that CK2α is actually found on the *PR1* promoter (Figure 10) especially after SA treatment giving evidence of co-localization of enzyme and substrate which in turn suggests that the events observed *in vitro* most likely happen on the *PR1* promoter in the plant nucleus as well.

Along with the knowledge that the POZ domain of NPR1 is able to restore DNA binding of phosphorylated TGA2 (Figure 8), the data we present in this study is sufficient to propose a modification of the existing model describing how TGA2 and NPR1 regulate transcription of the *PR1* promoter by introducing a novel molecular component in the form of protein kinase CK2.

5.1 A total of 13 amino acids within TGA2 are phosphorylated by CK2

Initially, it was concluded that 3 particular residues of TGA2 are phosphorylated by CK2 (Kang and Klessig, 2005). This turned out to be true but only part of the story, as there are a total of 13 targeted sites based on our mass spectrometry data. This discrepancy is not caused by incorrect data interpretation but rather by limitations of the methodology used. There, phosphorylation sites were identified using a set of TGA2 deletion or substitution mutants phosphorylated using radioactive ATP as a phosphate donor (Kang and Klessig, 2005). Very little radioactive phosphate was incorporated into mutants lacking the first 20 N-terminal amino acids and substitutions of Ser^{11}, Thr^{12} and Thr^{16} by

alanines also reduced the signal to a minimum. Therefore, it was assumed that these are the only residues targeted by CK2.

However, subtle clues indicating that this may not be completely true can be observed even in this previous study. For example, although the radioactive incorporation into the aforementioned mutants was minimal, there still was some signal detected. Additionally, a hint of a small signal decrease could be seen when Thr^4, Ser^5 and Thr^8 were substituted by alanines. Based on our results, this is attributed to the fact that Thr^8 is also phosphorylated. Furthermore, our work with TGA2 N-terminal deletion mutants shows that the speed of phosphorylation leading to a decrease in DNA binding is significantly reduced as more N-terminal amino acids are deleted. A relatively lower CK2 amount was sufficient to phosphorylate 95% of the wild type protein, but the efficiency was diminished to only 52% when the first 12 amino acids were deleted and a mutant lacking 24 amino acids displayed only 24% efficiency (Figure 4). Therefore, the beginning of the N-terminal domain seems to be important for efficient phosphorylation of the entire N-terminus by CK2 and the presence of numerous aspartates and glutamates commonly found in CK2 target sequences (Meggio et al., 1994) in this region would support this idea. The absence of these negatively charged residues could lower the affinity of TGA2 to CK2 and, therefore, slow down phosphorylation of subsequent target residues. Additionally, there could be a structural motif in this area the deletion of which could have such negative effects on CK2 recognition of TGA2.

Alternatively, the enzyme could phosphorylate sequentially, starting at the beginning of the N-terminus and proceeding inward. Keeping the majority of the N-terminal sequence untouched including the important aspartates and glutamates but replacing the phosphorylatable residues (as in the TGA2-3A or TGA2-3D mutants) could slightly alter the structure of this region and potentially slow down the enzyme resulting in lower phosphate incorporation as was seen by Kang and Klessig (2005).

In conclusion, the fact that phosphorylation is less efficient when TGA2 N-terminal deletion and substitution mutants are used, along with possible insufficient incubation time periods and lower incorporation of radioactive phosphate into proteins lacking multiple target residues in general most likely accounted for the low signal detected by Kang and Klessig (2005) causing them to miss the contribution of additional residues.

5.2 Loss of DNA binding is caused by phosphorylation of residues found in a multiserine motif of TGA2

The crucial ability of TGA transcription factors to bind DNA is mediated by their basic region/leucine zipper domain (bZIP). Proteins containing this domain are found in all eukaryotic species including plants. The bZIP domain is highly conserved among these species and is comprised of two distinct motifs: the N-terminal basic region and the C-terminal leucine zipper (Hurst, 1994).

As the name suggests, the basic region contains numerous basic (positively charged) amino acid residues. These interact with the negatively

charged nucleic acid. In most cases, bZIP transcription factors interact with palindromic binding sites formed on the DNA strands (Jakoby et al., 2002). For example, plant bZIP factors preferentially bind DNA sequences with an ACGT core and nucleotides flanking this sequence regulate binding specificity (Izawa et al., 1993). Downstream of the DNA-binding region is the leucine zipper motif responsible for protein dimerization. This intermolecular effect is achieved via a heptad repeat of leucines (Jakoby et al., 2002). This motif forms an α-helical protein structure where 3.5 residues are necessary to make one turn. Since every seventh amino acid is a leucine, all leucines forming this motif are oriented into the same direction on the α-helix. Thus, these leucines are capable of interacting via hydrophobic interactions with those found in the leucine zipper motif of another protein molecule resulting in protein dimerization.

Both the basic region and the leucine zipper take on the shape of α-helices (Ellenberger, 1994). Therefore, the resulting bZIP dimer is composed of two smoothly curving α-helices held together at the C-terminus by leucine zipper motifs forming a coiled-coil, while the N-terminus diverges and positions the relatively straight basic region helix into the major groove of the DNA molecule where it contacts both the nucleoside bases and the phosphodiester backbone (Ellenberger, 1994).

In a study in 2006, Amoutzias et al. focused on how DNA binding in bZIP dimerization proteins can be controlled by reduction/oxidation or phosphorylation. Alignment of basic region sequences of bZIP transcription factors found in human, fly, cnidaria and fungus uncovered that most of these proteins contain

either a serine or cysteine in position 19. Previous studies showed that Ser^{19} is phosphorylated in the Basic leucine zipper transcriptional factor ATF-like protein and the CCAAT-enhancer binding protein and that this post-translational modification attenuates DNA binding in both cases (Mahoney et al., 1992; Deppmann et al., 2003). Furthermore, when Cys^{19} is oxidized in activator protein 1, its DNA binding capabilities are also abolished (Abate et al., 1990). Therefore, it was suggested that modifications of the residue in position 19 of the basic region are responsible for regulating DNA binding of bZIP transcription factors (Amoutzias et al., 2006).

Nevertheless, this is probably not true in the case of plant bZIP proteins which were not investigated in Amoutzias' study (2006). Mass analysis of phosphorylated TGA2 from Arabidopsis did not detect any modification within the basic region (Figure 5) despite the fact that there is one serine and one threonine located in this area. Additionally, TGA1 is not phosphorylated by CK2 (Kang and Klessig, 2005) despite the fact that it contains the same conserved serine within the basic region as TGA2. However, DNA binding of TGA1 was reportedly promoted via oxidation using S-nitrosoglutathione (Lindermeyr et al., 2010) but TGA1 does not contain a single cysteine in its basic region meaning that any regulatory oxidation events occur on cysteines found outside this region. Therefore, it seems that bZIP regulation suggested by Amoutzias et al. (2006) cannot be extrapolated to the TGA plant system. The reason for this may be found in the complexity of plant primary and secondary metabolism which needs additional regulation compared to, for example, that of humans. In Arabidopsis, a

total of 81 proven or putative bZIP genes were identified (Riechmann et al., 2000), which is approximately 4 times more than in other eukaryotic organisms including yeast, worm or human (Jakoby et al., 2002). Many of these factors, including the TGAs, have been identified only in several plant species and control processes unique to the plant kingdom (Alvez et al., 2013).

Although our data show that there is not a single phosphorylated residue in the basic region, there is one (Thr^{80}) found in the leucine zipper motif adjacent to the central leucine residue (Figure 5). In theory, an addition of a negatively charged phosphate in close proximity of this leucine in each of the two interacting protein molecules could disrupt dimerization as well as DNA binding. This hypothesis was tested using leucine zipper deletion mutants of TGA2 (Figure 6). Although deletion of the majority of the leucine zipper motif including Thr^{80} resulted in decreased affinity of the TGA2Δ73-92 mutant to DNA, this protein was still capable of binding DNA to a certain extent (Figure 6C). Kinase treatment disrupted this interaction suggesting that the missing threonine does not control this event.

On the other hand, EMSA results of TGA2 N-terminal deletion mutants clearly show that a mutant lacking the first 24 amino acids loses its affinity to DNA after phosphorylation, whereas a mutant lacking the first 43 amino acids does not despite the fact that it is targeted by the kinase (Figure 3). This would implicate the residues between position 24 and 43 as those responsible for regulating the DNA binding ability of the bZIP domain. The presence of several aspartates also suggests that this site is recognized by CK2. Mass analysis

revealed that 5 residues in this area are phosphorylated; Thr^{30}, Ser^{33}, Ser^{35}, Ser^{36} and Ser^{39} (Figure 5). Therefore, we created the TGA2-5A mutant where all these residues were replaced by alanines. This protein was able to bind DNA and this did not change when CK2 was added to the sample (Figure 7). This result conclusively demonstrates that this area is in fact the crucial regulatory region affected by phosphorylation. However, it is yet unclear if all targeted residues or just a subset of these are needed for successfully regulating DNA binding.

Sequence alignment of TGA factors reveals that Thr^{30} is not conserved within the TGA-II clade as TGA5 and TGA6 do not contain this residue (Supplemental Figure 5). However, the remaining serines are found in all three proteins, thus the conserved SDSSDRS sequence found in all these three TGAs is most likely responsible for regulation. Identification of the precise residues necessary to abolish interaction with DNA using substitution mutants may prove to be difficult or even practically impossible as the close proximity of these residues (especially Ser^{35} and Ser^{36}) could mean that the kinase may not differentiate among these targets and phosphorylation of any of these or any of their combination will result in the same effect.

It would be plausible that due to the close proximity of this multiserine motif to the beginning of the basic region responsible for binding to DNA (residue 44 in TGA2), any modification involving the addition of negatively charged phosphates could lead to repulsion of this protein domain away from the negatively charged phosphodiester backbone of DNA or could cause

conformational changes within this area thus negatively affecting interaction with the nucleic acid.

Interestingly, very similar motifs are also found just upstream of the basic region in TGA2 paralogs in *Oryza sativa* and *Nicotiana tabacum* (Supplemental Figure 6). Transcription factor TGA2.1 from tobacco contains an SSDKS motif while the TGA2 paralog in rice has a SNSSDRS sequence just upstream of its basic domain. The fact that this motif occurs in TGA2s of various dicot (Arabidopsis and tobacco) and even monocot (rice) species implicates that this particular multiserine motif is essential for proper regulation and function of these proteins *in vivo* as it has been at least partially maintained throughout separate evolution of these plant species.

In conclusion, our results show the multiserine motif found between position 33 and 39 of TGA2 is responsible for regulation of this transcription factor's DNA binding abilities via CK2 phosphorylation.

5.3 Protein deoligomerization is attributed to phosphorylation of Thr^{80} found within the leucine zipper motif

Stoichiometry changes of TGA2 protein homocomplexes seem to be an inherent part of their role in regulating the onset of systemic acquired resistance. Gel filtration analyses using recombinant Arabidopsis TGA2 uncovered that this protein forms higher-order complexes formed by 40 or more TGA2 molecules *in vitro* (Boyle et al., 2009). Furthermore, these large complexes are capable of interacting with DNA without changing their stoichiometry. Remarkably, addition

of the crucial SAR co-regulator NPR1 enables the formation of smaller protein complexes, more specifically a TGA2-NPR1-DNA enhanceosome complex occurring in a 2:2:1 or 2:1:1 ratio. These results were supported by *in vivo* experiments when TGA2 oligomers were detected on the *PR1* promoter prior to but not after SA treatment of Arabidopsis tissue (Boyle et al., 2009) and by the fact that the TGA2-NPR1 enhanceosome is formed on the promoter after SA application (Rochon et al., 2006).

However, the reason for TGA2 oligomer disassembly remained unknown until we performed gel filtration experiments using TGA2 phosphorylated by CK2 (Figure 2). Here, we observed that although the majority of phosphorylated protein remained as an oligomer, a smaller portion was detected in its monomeric form. A similar deoligomerization effect was also observed when N-terminal deletion mutants of TGA2 were analysed after being incubated with CK2 (Figure 3). Therefore, it is most likely phosphorylation that partially breaks down the oligomeric complex providing solitary TGA2 molecules that could be later used for creating an enhanceosome with NPR1 on the *PR1* promoter.

Interestingly, triple substitutions of Ser^{11}, Thr^{12} and Thr^{16} in the N-terminus by aspartates (TGA2-3D) or alanines (TGA2-3A) also resulted in formation of smaller complexes including monomers, dimers, trimers and tetramers without the use of the kinase (Figure 2). However, such amino acid alteration can have a profound impact of the overall protein structure via alteration of the hydrogen bond network. It seems that such changes negatively affect TGA2 protein-protein interaction domains either by lowering the strength of these interactions or due to

sterical hindrance of the deformed N-terminal structure caused by altering its amino acid composition. The final result in either case would be the inability of some protein molecules to be incorporated into higher-order complexes.

Using gel filtration data from various TGA2 deletion mutants, we were able to narrow down the region that is responsible for TGA2 disassembly. As mentioned previously, all TGA2 N-terminal deletion mutants (Δ12TGA2, Δ24TGA2 and Δ43TGA2) formed smaller protein complexes or monomers after CK2 treatment (Figure 3). Therefore, it is logical that the protein-protein interaction domain affected by the kinase is not found in the N-terminus, which is completely missing in the Δ43TGA2 protein, but further down in the direction of the C-terminus. The only other known protein-protein interaction domain in this area is the leucine zipper, although the presence of another dimerizing motif cannot be ruled out as the Δ93TGA2 mutant lacking the N-terminus and the bZIP domain is capable of forming dimers as well as monomers (Boyle et al., 2009).

Our mass analysis of phosphorylated TGA2 revealed that Thr^{80} is targeted by CK2, a residue that is directly next to the central leucine of the leucine zipper motif (Figure 5). As discussed previously, incorporation of a phosphate group with a strong negative charge in the close vicinity of the hydrophobic leucine could disrupt previously formed interactions among the leucine zippers of two TGA2 molecules. Although phosphorylation of this residue doesn't seem to regulate the DNA binding abilities of TGA2 (Figure 6), it could be responsible for protein deoligomerization. Once again, gel filtration analysis was performed to analyze the TGA2 mutant lacking the majority of the leucine zipper including

Thr^{80} (TGA2Δ73-92; Figure 6). No smaller complexes or monomers were detected when this protein was phosphorylated despite the fact that this protein is targeted by the kinase as can be seen by the migration shift of phosphorylated TGA2Δ73-93 on a gel compared to the protein that was not incubated with CK2 (Figure 6). This clearly implicates Thr^{80} as the residue responsible for TGA2 complex disassembly.

Comparison of amino acid sequences of various TGA factors show that the Thr^{80} residue is conserved among the TGA2, TGA5 and TGA6 which are regulated by CK2, but not in TGA1 or TGA4 (Supplemental Figure 5) which do not seem to be targeted by CK2 (Kang and Klessig, 2005). In the case of the latter, threonine is replaced by an isoleucine. Interestingly, the tobacco TGA2 homolog has a serine in this position (Supplemental Figure 6). Conversely, TGA2 found in rice has an alanine which is, however, neighboured by a serine in the subsequent position. This information suggests that the presence of a phosphorylatable residue in this area of the leucine zipper is likely important for the transcription factor's function as it has been conserved throughout evolution. This would also mean that a similar deoligomerization effect triggered by CK2 is highly likely in the case of other Arabidopsis clade-II TGAs and possibly even in the TGA2 homologs found in other plant organisms. However, this hypothesis would have to be tested as Thr^{80} is not conserved in rice or tobacco TGA2.

In the past, protein phosphorylation has been linked with the change of stoichiometry of protein complexes on multiple occasions although in most cases it is simple protein dimer formation or disassembly (Liu et al., 2012; Marie et al.,

2000). Nevertheless, several studies observed changes in larger protein complexes as well. For instance, Roigot et al. (2014) studied the human oligomeric Arg-binding Protein 2 and found that its complexes are destabilized by tyrosine phosphorylation. The resulting smaller complexes have altered affinity to interacting molecular partners of this protein and thus these changes regulate its function within cytoskeleton associated signal transduction. Another example of protein deoligomerization would be that of the small Heat Shock Protein 27 which is a chaperone pertaining to responses to oxidative stress (Rogalla et al., 1999). This protein also forms oligomers but phosphorylation by a mitogen-activated protein kinase disassembles these complexes into tetramers which down-regulates their activity.

However, very few examples of altered protein stoichiometry triggered by CK2 exist. One such example is the phosphoprotein P of Chandipura virus which normally forms monomers but dimerizes after being phosphorylated by this kinase, an event activating this protein in the transcriptional process (Raha et al., 2000). Additionally, the Histone Deacetylase 1 and 2 enzymes involved in chromatin remodeling and gene expression regulation are capable of forming homo or heterodimers, a necessary prerequisite for enzymatic activity (Khan et al., 2013). Phosphorylation by CK2 is crucial for dissociation of the dimers which alters their function during mitosis. Finally, Lischka et al. (2007) found that an mRNA transport factor called UL69 from the human cytomegalovirus requires its central domain for self-association. The same domain also binds the CK2 kinase;

however, the effect of phosphorylation on protein complex formation was not investigated.

It seems that our study is the first to show that CK2 phosphorylation is responsible for deoligomerization of protein homocomplexes as well as provide a possible mechanism for this event: incorporation of a phosphate moiety into the hydrophobic leucine zipper could disrupt its function as a protein-protein interaction domain resulting in the release of smaller protein complexes or monomers.

5.4 Arabidopsis CK2α is located on the *PR1* promoter and its recombinant form phosphorylates TGA2

Finally, the relevance of all presented results obtained *in vitro* needed to be supported by *in vivo* data. It is clear that phosphorylated TGA2 can be obtained and studied in a laboratory setting but does this modification occur also in the plant? Circumstantial evidence has been presented in the past including the use of Arabidopsis tissue lysates to phosphorylate TGA2, the up-regulation of Arabidopsis and tobacco CK2 activity after SA application, as well as the lower relative induction of reporter gene expression under the control of a portion of the *PR1* promoter after SA treatment when a CK2 inhibitor was added (Kang and Klessig, 2005; Hildago et al, 2001).

To address the role of CK2 in SAR, we performed ChIP experiments which are able to determine if a particular protein is interacting with a particular area of the genome. Using a PCR primer pair targeting the area of the *PR1*

promoter to which the TGA transcription factors bind and a second pair targeting an area where no TGA-binding sequences are located, we were able to determine that CK2 is in fact recruited to the promoter in the same area as where the TGAs are located and that there is more CK2 recruited to this region after SA treatment (Figure 10). This result proves that both the kinase and the targeted transcription factor are found at the *PR1* promoter in the same place and at the same time which, therefore, gives strong evidence that the reaction we observed *in vitro* is likely occurring in the plant as well.

For the ChIP procedure, we used a polyclonal anti-CK2 antibody raised against an epitope corresponding to amino acids 2-320 of the human CK2α. This antibody was successful in detecting Arabidopsis CK2α (Supplemental Figure 4) and pulling down this protein in the ChIP experiment (Figure 10). This was enabled by the sequence similarity of the CK2α isoforms which, in general, are highly conserved among species (Guerra and Issinger, 1999). The major differences between the human CK2α and, for example, Arabidopsis CK2α1 are that the former has an additional C-terminal sequence, whereas the latter usually has an N-terminal extension (Supplemental Figure 3). Without these, the cores of the two enzymes have a 72.2% sequence identity. Moreover, the number of similar residues is also quite high. Similar residues are two different amino acid residues with the same characteristics in a particular site (for example, having a hydrophobic leucine in one homolog and an equally hydrophobic isoleucine in the other). Therefore, the final sequence similarity is up to 91% which is the reason why the various isoforms can be detected by the antibody we used.

The *in vivo* result shows that CK2α is localized to the same area as TGA2. We also tested if these two proteins could physically interact by using pull-down experiments where HisTrap column-bound His-tagged proteins were used to retain the Strep-tagged Arabidopsis CK2α1 (Figure 9). Both full length TGA2 and its N-terminal deletion mutant lacking the first 43 residues were able to bind this recombinant enzyme. However, His-tagged NPR1 POZ or the column resin without any protein was not. These results tell us two important facts. First of all, Arabidopsis CK2α1 is able to interact with the TGA2 mutant lacking the entire N-terminus. Therefore, the enzyme is bound to TGA2 by a different domain. Secondly, the enzyme does not interact with NPR1 POZ meaning that the restoration of DNA binding of phosphorylated TGA2 (Figure 8) is not mediated by the interaction of TGA2-CK2-POZ and that CK2 is not recruited to TGA2 via POZ.

CK2 has been shown to target many bZIP transcription factors (Yamaguchi et al., 1998). Use of the Activating Transcription Factor 1 protein and its deletion mutants revealed that CK2α directly binds to this bZIP protein via the basic region of the bZIP domain (Yamaguchi et al., 1998). Deletion of this domain markedly reduces phosphorylation. Therefore, the kinase is recruited to DNA via the transcription factor and not by its own DNA binding domain. As the basic region is highly conserved among bZIP transcription factors many of which have been shown to be targeted by CK2 (Yamaguchi et al., 1998), it is highly probable that it is the basic region of TGA2 that interacts with the enzyme. Our results clearly show that deletion of the N-terminus or deletion of the leucine zipper does

not stop phosphorylation by CK2 (Figure 3 and 6) leaving the basic region as the most likely candidate for docking CK2. Interestingly, this is also the only region without a single phosphorylation site (Figure 5).

As Arabidopsis CK2α1 interacts with TGA2, we were interested if the plant isoform also affects TGA2's DNA binding affinity. In contrast to the tetrameric CK2 holoenzyme isolated from humans, animals and yeast, the plant homolog can be found as either a CK2α monomer or the CK2 tetramer formed by two α and two β subunits (Klimczak et al., 1995). Interestingly, both the plant CK2α monomer and the recombinant human CK2α alone possess enzymatic activity, although interaction with the respective CK2β protein stimulates their activity in both cases (Klimczak et al., 1995, Tuazon and Traugh, 1991).

Our goal was to test the enzyme activity of the *E. coli* produced and affinity purified recombinant CK2α originating from Arabidopsis and to examine its effect on DNA binding of TGA2. As seen in Figure 9, the CK2α1 alone is capable of phosphorylating TGA2 which decreases this transcription factor's DNA binding ability. This confirms that both the human and plant isoforms have a similar effect on this transcription factor. Additionally, the Arabidopsis CK2α1 can use both ATP and GTP, a typical CK2 trait (Hathaway and Traugh, 1982).

Further evidence of similar characteristics of the recombinant human and plant CK2α was provided by experiments when this protein was incubated along with ATP (Figure 9). The fact that the amount of phosphorylated recombinant CK2α1 increases with time hinted possible autophosphorylation. This event has

been described in the human CK2α protein (Donella-Deana et al., 2001). Amazingly, this protein is capable of phosphorylating other CK2α molecules on Tyr^{182} providing a rare example of tyrosine phosphorylation by CK2. Interestingly, this modification is reversible and prevented by the presence of regulatory β subunits. The effect of this modification on enzymatic activity was not tested but it was hypothesized that this modification would weaken the interaction between the activation loop and N-terminus of CK2α thus decreasing its activity towards other protein targets. The Arabidopsis CK2α1 does contain this tyrosine (Supplemental Figure 3); therefore, it is most likely the same residue that is modified in the plant isoform. This result clearly demonstrates that CK2α isoforms from the two different organisms behave in a very similar manner.

The presented data were obtained using only the Arabidopsis CK2α1, not the other isoforms. Contrary to other species, plants have evolved high numbers of both CK2α and CK2β isoforms. In Arabidopsis, there are a total of four CK2α and also four CK2β proteins (Salinas et al., 2006; Velez-Bermudez et al., 2011). A study by Mulekar et al. (2011) investigated the roles of various Arabidopsis CK2α isoforms, with the exception of the chloroplastic α4, using single, double and triple knock-out mutants and observed that only the triple mutant displayed significantly different traits and gene expression levels. Therefore, it seems that the three α isoforms are redundant in Arabidopsis meaning that one could function in the same way as the others and thus phosphorylate similar, if not identical, sites of target proteins. However, the matter of regulation of these active subunits is complicated by the fact that there are multiple regulatory β

proteins which can also interact with each other in various combinations with various affinities (Riera et al., 2001b). This was the main reason why we tested only one α isoform without the use of any β subunits as the amount of possible combinations is simply too high.

5.5 New derepression step identified in TGA2's role in SAR

TGA2 and its role in SAR have been studied extensively, however, only one study focusing on the molecular aspects of this protein was capable of obtaining sufficient data to be able to construct a model of how this transcription factor regulates the *PR1* gene (Boyle et al., 2009). This model showed that TGA2 is found at the *PR1* promoter in the form of large complex of unknown stoichiometry (also depicted in Figure 11A). After SAR initiation via pathogen infection or exogenous SA application, this protein complex disassembles and a TGA2 dimer is capable of interacting with an NPR1 dimer forming an enhanceosome activating *PR1* transcription. This mechanism explained how this transcription factor can act as both a repressor and activator at various conditions. Nevertheless, the one weak point of this model was that no presented *in vitro* or *in vivo* data could fully unveil how the TGA2 complex deoligomerizes. The authors hypothesized that after SA stimulation, the POZ domain of NPR1 could either act as a crowbar chaperone to disassemble the TGA2 complex or evict the oligomer from the promoter by interacting with TGA2 dimers (Boyle et al., 2009).

Our results fully resolve this issue by showing that it is TGA2 phosphorylation by CK2 that assists in this event. Large TGA2 complexes are

capable of interacting with DNA *in vitro* and are found on the *PR1* promoter prior to SA treatment of Arabidopsis tissue (Boyle et al., 2009). Protein kinase CK2, which is present on the *PR1* promoter in the same region as are the TGA binding sites (Figure 10) and whose activity is up-regulated by SA application (Kang and Klessig, 2005) which could possibly be as a result of its translocation, phosphorylates the transcription factor evicting the TGA2 complexes from the promoter. This is due to the crucial modification of the multiserine motif within TGA2's N-terminus which abolishes this protein's DNA binding ability (Figure 7). Additionally, phosphorylation within the leucine zipper region of TGA2 most likely disrupts the function of this protein-protein interaction domain leading to the release of smaller TGA2 complexes and even monomers (Figures 2 and 3). These free molecules could be easily recognized by NPR1 molecules which are also released from larger NPR1 complexes after direct interaction with SA (Wu et al., 2013). TGA2 interacts with the POZ domain of NPR1 via its N-terminal domain (Boyle et al., 2009) and although much phosphorylation is targeted to this TGA2 area, it has little effect on the resulting protein-protein interaction as the POZ domain is not only capable of interacting with phosphorylated TGA2 but also restores the DNA binding ability of phosphorylated TGA2 (Figure 8).

Therefore, we propose the existence of an additional derepression step in the model previously published by Boyle et al. (2009). As seen in Figure 11B, the TGA2 oligomer is released from the promoter due to phosphorylation by CK2 while releasing some TGA2 monomers. This would basically derepress the gene

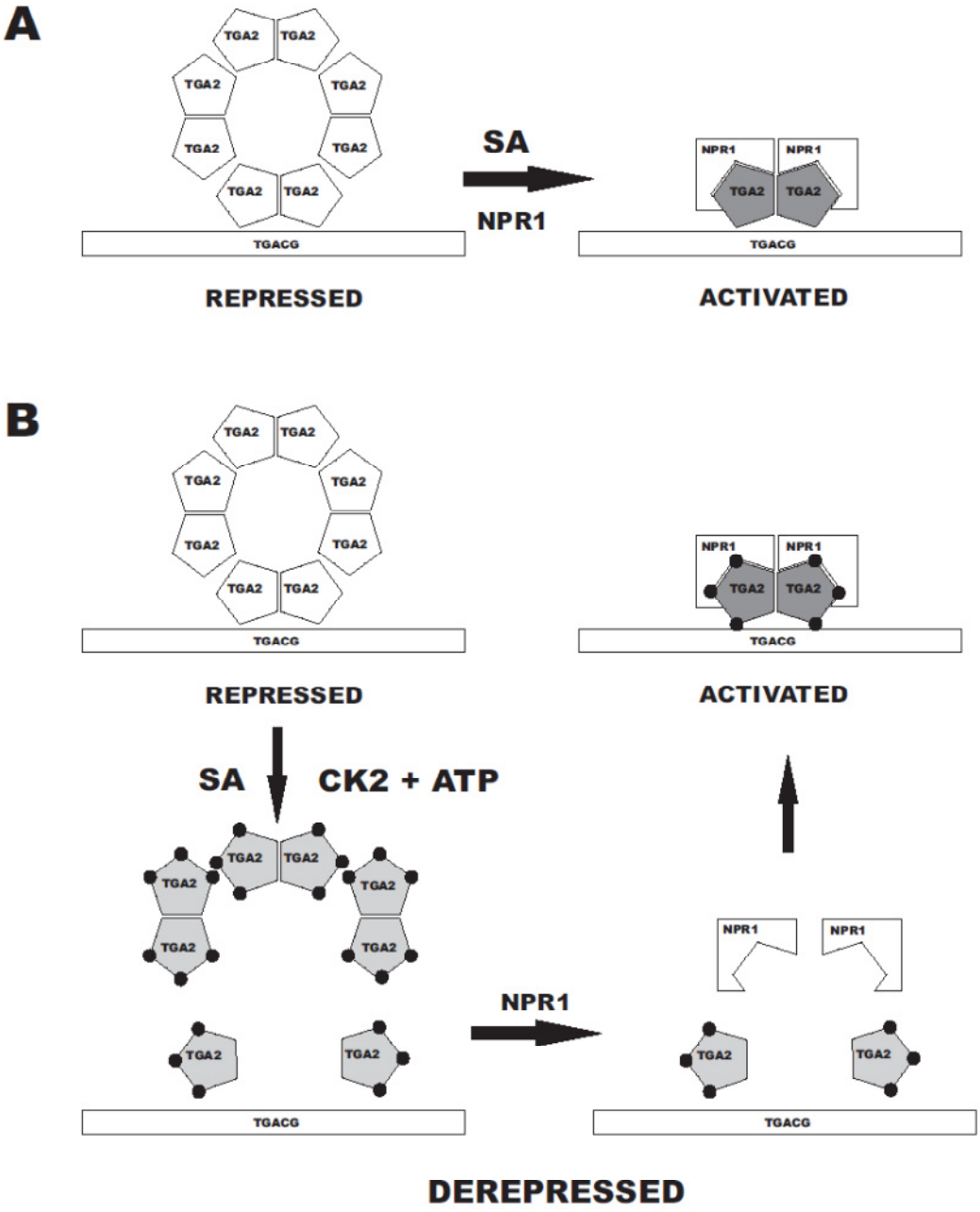

Figure 11. The accepted model depicting TGA2 regulation of *PR1* and its proposed modification.

(A) The previously published model depicting how TGA2 forms an oligomer (white) which represses the *PR1* gene prior to SA accumulation and the activation of the same gene by a TGA2 dimer (dark grey) after interaction with

NPR1 (modified from Boyle et al., 2009). The TGACG nucleotide sequence is depicted within the *PR1* promoter (white box).

(B) The modified model proposed in our study depicting how CK2 impacts *PR1* regulation. A TGA2 oligomer (white) represses *PR1* expression. After SA accumulation, CK2 phosphorylates TGA2 (light grey with black circles indicating additional phosphates) which is released from the TGA binding sequence of the *PR1* promoter (white box) and partially deoligomerizes. In this derepressed state, no TGA2 molecules physically interact with the promoter. The free TGA2 monomers are recognized by NPR1, interact together and form an enhanceosome. Therefore, at this point TGA2 acts as an activator (dark grey) and up-regulates *PR1* expression.

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as there would be no TGA2 to repress its expression. Subsequently, the free TGA2 monomers would readily interact with NPR1 forming the enhanceosome necessary for *PR1* activation.

In conclusion, the presented model modification would directly link the CK2 to the regulatory machinery of the *PR1* gene and finally resolve the unanswered question of TGA2 complex disassembly. Additionally, this model could be extrapolated to other Arabidopsis TGA transcription factors (TGA5 and TGA6) as well as to TGA2 homologs found in other plant species (rice and tobacco) as these proteins share similarities in their amino acid sequences and, in several instances (Hildago et al., 2001), have been shown to be targeted by CK2.

CHAPTER 6 - CONCLUSIONS AND FUTURE RESEARCH

Despite the fact that a previous study investigated the effect of protein kinase CK2 on the TGA2 transcription factor, we provide an in-depth analysis of this event, rectify the previously published inaccuracies and put the entire event into a broader perspective within systemic acquired resistance (SAR). Not only did our work uncover multiple new phosphorylation sites within TGA2, we were also successful in narrowing down the regulatory region affecting DNA binding of this factor and the residue most likely to be responsible for oligomer disassembly. As a result, we proposed a modification of the currently accepted model of how the model plant defence gene *PR1* is regulated via TGA2 and NPR1 by adding a crucial phosphorylation step necessary to evict and disperse repressing TGA2 oligomers and provide TGA2 monomers capable of forming an activating complex with NPR1.

Although the data we present solve many unresolved issues, it also leads to several questions that should be addressed in subsequent research. For example, our study focused on the human and Arabidopsis CK2α1 *in vitro* and all CK2α isoforms *in vivo*. This raises the question if the catalytic kinase isoforms function similarly or not. A previous study (Mulekar et al., 2011) generated multiple CK2α gene knock-out mutants including single, double and triple mutants. Using these plants, they observed that the three CK2α genes (excluding the fourth chloroplastic gene which is most likely essential for plant viability and thus cannot be knocked out) seem to have an additive effect on many traits and events in Arabidopsis, such as flowering, growth and development or stress

responses. These mutants could be used to great effect in future chromatin immunoprecipitation experiments to see if all 3 CK2α isoforms have a similar function *in vivo* during plant responses to biotrophic pathogens. Based on the findings of Mulekar et al. (2011), it can be hypothesized that the isoforms are most likely redundant. Nevertheless, this hypothesis must be first tested as differential compartmentalization, gene expression or separate recruitment of these enzymes to a particular DNA sequence cannot be ruled out. Furthermore, should only one CK2α isoform be responsible for TGA2 phosphorylation *in vivo*, an effort to understand its uniqueness would commence. Differential pairing with regulatory CK2β isoforms, the interaction with another protein or the ability to perceive the salicylic acid (SA) signal directly or indirectly could all be possible reasons.

Interestingly, it has been shown that CK2 is active prior to SA accumulation and its activity is further increased after SAR is triggered (Kang and Klessig, 2005). Therefore, there must be a mechanism by which the kinase is activated. Our results suggest that there is transport of the enzyme to the promoter after SA; however, these results don't provide any information on enzymatic activity. Due to the rapid onset of activity increase within several minutes after SA treatment, it is most likely a post-translational modification, such as phosphorylation, of the kinase or interaction with an unknown activator that increases the overall enzymatic activity, not novel CK2 protein synthesis. This would suggest the existence of a possible additional SA receptor, since NPR1/3/4 (the identified SA receptors) haven’t been shown to trigger kinase

cascades or directly interact with CK2. However, these are just hypotheses that would have to be investigated using a method like chromatin immunoprecipitation to completely understand this event.

Based on our results and those of previous studies, the TGA2 transcription factor is capable of forming multiple intermolecular protein-protein interactions (e.g., with CK2, NPR1's POZ domain, and other TGA2 molecules), as well as intramolecular interactions (the N-terminus with the remainder of the TGA2 protein). It would be highly valuable to obtain the exact 3D structure of this protein to be able to address more specifically how these interactions are formed and potentially how does phosphorylation affect the overall structure. However, this may prove to be difficult as TGA2 is a fairly large protein to be analysed using nuclear magnetic resonance and precipitates in solution when purified to higher concentrations, which would hinder any crystallography efforts. Testing various conditions in order to diminish precipitation would most likely be the first step in this endeavour to determine the structure of this transcription factor.

Amino acid sequence alignments of various TGA transcription factors show that the N-terminal regulatory sequence responsible for abolishing DNA binding are conserved among the Arabidopsis, rice and tobacco TGA2 homologs. Additionally, all these proteins also have a phosphorylatable residue within their leucine zipper domain. These similarities would suggest that the function and phosphorylation modification of TGA2 during pathogen attack is conserved among various plant species. Testing DNA binding and protein complex

redistribution after phosphorylation by CK2 of TGA2 from rice and tobacco would support or reject this hypothesis.

Fully understanding the relevance of these components of SAR activation, especially in widely-cultivated crops, is highly important not just from the basic research point of view, but also in applied research. Genetic manipulation of crop plants designed to increase disease resistance against a wide array of potential pathogens would result in lower pesticide use. On the other hand, permanent and strong up-regulation of these defences would be ill-advised as the plant's energy would be diverted from growth and seed or fruit production. Therefore, more subtle modifications of the defence pathways will most likely be key to this engineering endeavour and for these we need to understand in detail how plant defence mechanisms are up-regulated. Our study provides sufficient detail and biological relevance to support this effort.

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APPENDIX - SUPPLEMENTAL FIGURES

Supplemental Figure 1. Operational parameters and standard curve used for calibration of the S300 gel filtration column.

V_0 (void volume of the column evaluated with Blue Dextran 2000) = 35.52 ml

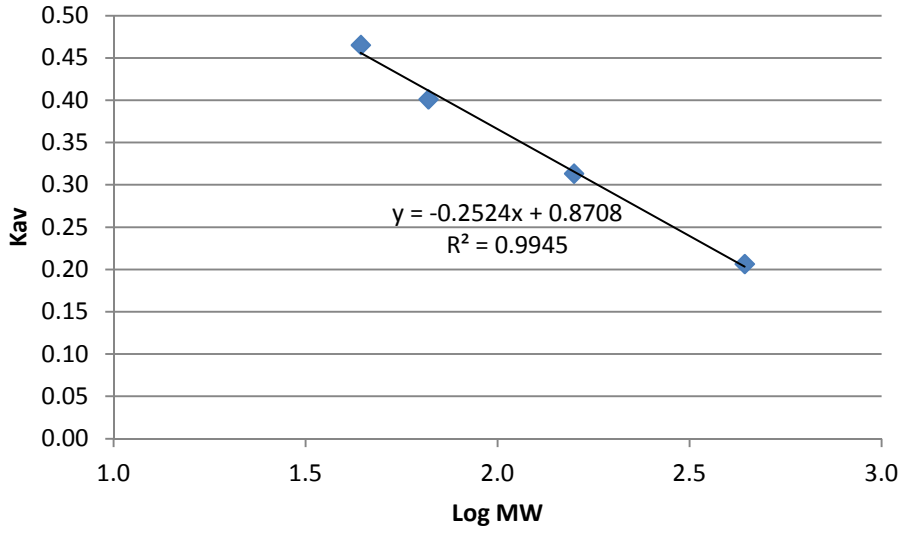
V_t (total bed volume of the column) = 104.5 ml

V_e (protein elution volume)

K_{av} (gel phase distribution coefficient)

$$K_{av} = \frac{V_e - V_0}{V_t - V_0}$$

Protein	MW (kDa)	Log MW	V_e (ml)	K_{av}
Ferritin	440	2.6435	49.77	0.2066
Aldolase	158	2.1987	57.14	0.3134
Bovine seum albumin	66	1.8195	63.2	0.4013
Ovalbumin	44	1.6435	67.62	0.4654



Supplemental Figure 2. Mass analysis of phosphorylated TGA2.


81
QLEQELQRAR QQGVFISGTG DQAHSTGGNG ALAFDAEHSR WLEEKNKQMN ELRSALNAHA GDSELRIIVD GVMAHYEELF


161
RIKSNAAKND VFHLLSGMWK TPAERCFLWL GGPRSSELLK LLANQLEPMT ERQLMGINNL QQTSQQAEDA LSQGMESLQQ

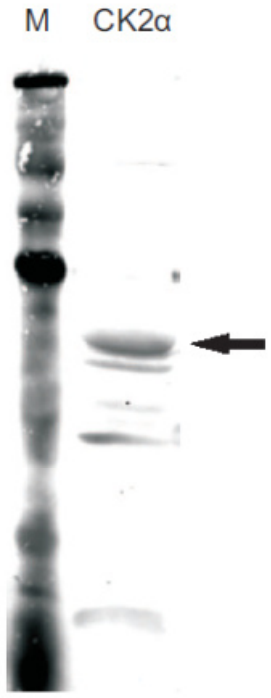

241
SLADTLSSGT LGSSSSGNVA SYMGCMAMAM GKLGTLEGPI ROADNLRLOT LOOMIRVLTT ROSARALLAI HDYPSRLRAL

Supplemental Figure 2. Visual representation of mass analysis data. The TGA2 protein sequence is displayed and modified residues are highlighted by various colors depending on modification (see legend). Below the sequence are the detected protein fragments (in green), their modifications are also highlighted. Residues phosphorylated by CK2 are in magenta or pink.

Supplemental Figure 3. Sequence alignment of CK2α homologs from *Arabidopsis thaliana* and human.

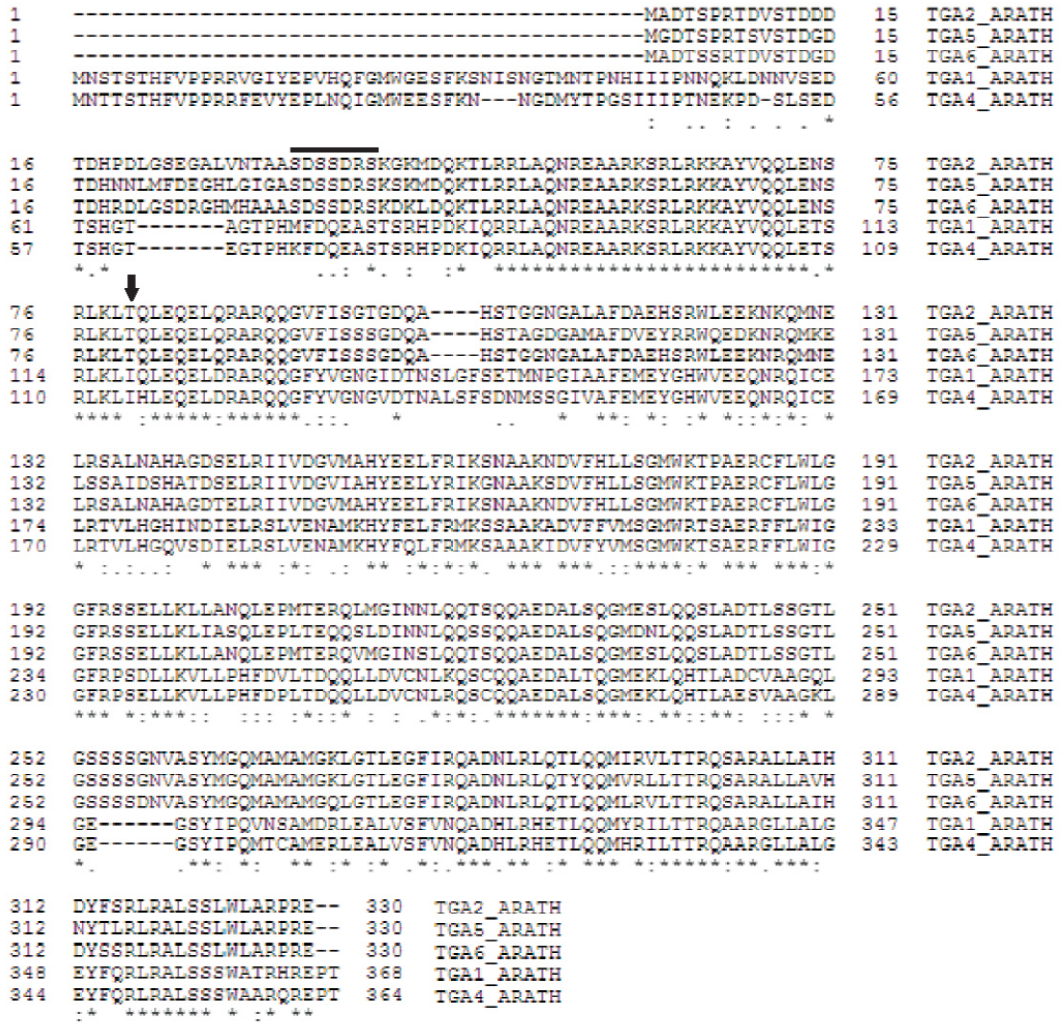
Supplemental Figure 3. Amino acid alignment of CK2α isoforms (CSK2) originating from *Arabidopsis thaliana* (ARATH), and the human homolog (HUMAN). The black arrow points to Thr^{182} of the human isoform which was found to be autophosphorylated. The (*) below the aligned sequences indicates identical residues, (:) indicates residues with strongly similar properties and (.) indicates residues with weakly similar properties. Alignment was created using the ClustalO program using sequences from the UniProtKB database.

Supplemental Figure 4. Test of anti-CK2α antibody specificity.



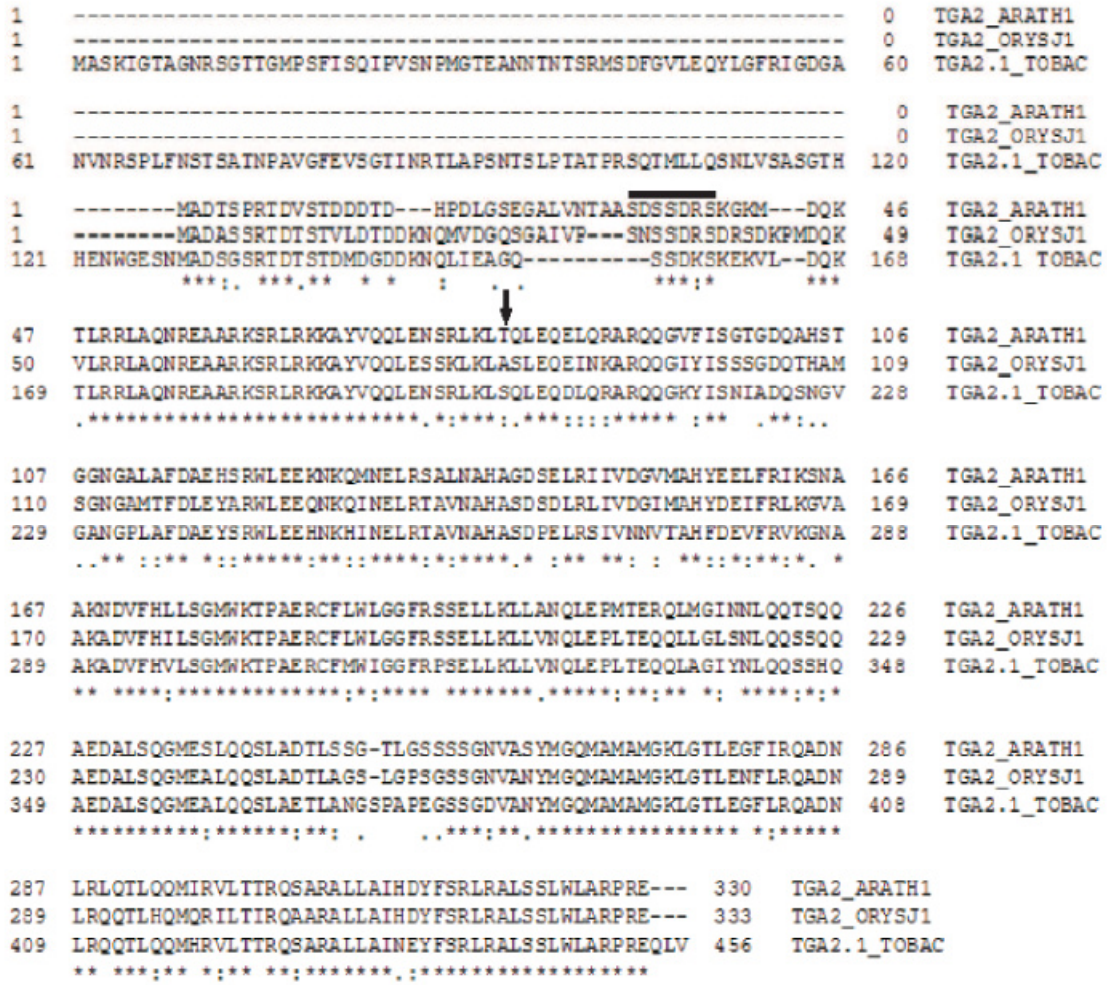
Supplemental Figure 4. Western blot analysis of an electrophoresed crude cell lysate of *E. coli* cells producing Strep-tagged Arabidopsis CK2α detected by the anti-CK2α antibody. The protein detected was the CK2α (black arrow) along with smaller proteins, which are most likely products of CK2 degradation by bacterial proteases. Lane M contains the protein standard marker. This analysis shows that the polyclonal anti-CK2α antibody is capable of specifically detecting Arabidopsis CK2α.

Supplemental Figure 5. Sequence alignment of clade I and II TGA transcription factors from *Arabidopsis thaliana.*



Supplemental Figure 5. Amino acid alignment of TGA2, TGA5, and TGA6 from clade II and TGA1 and TGA4 from clade I. All these proteins originate from *Arabidopsis thaliana* (ARATH). The black box indicates the multiserine SDSSDRS motif found only in clade II TGAs and the black arrow points to Thr^{80} in the leucine zipper motif. The (*) below the aligned sequences indicates identical residues, (:) indicates residues with strongly similar properties and (.) indicates residues with weakly similar properties. Alignment was created using the Clustalo program using sequences from the UniProtKB database.

Supplemental Figure 6. Sequence alignment of TGA2 transcription factor homologs from *Arabidopsis thaliana, Oryza sativa* and *Nicotiana tabacum.*



Supplemental Figure 6. Amino acid alignment of TGA2 homologs originating from *Arabidopsis thaliana* (ARATH), *Oryza sativa* (ORYSJI) and *Nicotiana tabacum* (TOBAC). The black box indicates the multiserine SDSSDRS motif or its variations and the black arrow points to Thr^{80} found in the Arabidopsis TGA2 leucine zipper motif. The (*) below the aligned sequences indicates identical residues, (:) indicates residues with strongly similar properties and (.) indicates residues with weakly similar properties. Alignment was created using the Clustalo program using sequences from the UniProtKB database.